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Regulation of angiotensinogen in adipocytes by polyunsaturated fatty acids

Sarah Jean Fletcher

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To the Graduate Council:

I am submitting herewith a thesis written by Sarah Jean Fletcher entitled "Regulation of angiotensinogen in adipocytes by polyunsaturated fatty acids." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.

Naima Moustaid-Moussa, Major Professor

We have read this thesis and recommend its acceptance:

Brynn H. Voy, Arnold M. Saxton, Guoxun Chen

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Regulation of angiotensinogen in adipocytes by polyunsaturated fatty acids

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Sarah Jean Fletcher
May 2010

Dedication

For Chad Aaron Melton, for a million reasons. Thank you for coming with me.

And for my parents for being my rock. You've done everything right.

Acknowledgements

I would like to thank those who have helped me, advised me and educated me throughout this process. I am eternally grateful for my advisor and mentor Dr. Naima Moustaid-Moussa. This project would not have been possible without her support and advice. I would also like to thank the other members of my lab, Nishan Kalupahana, Suzanne Booker, Rachael Hadidsaz, Nalin Siriwardhana, Morvarid Soltani-Bejnood, Lorin Hall and Jeffery Morris. I am extremely appreciative of the members of my thesis committee for their advice and suggestions as well as for their valuable time. I owe a debt of gratitude to my graduate program, Genome Science and Technology, for providing me with this opportunity and for educating me and supporting my research. Finally, I would like to thank my family and friends for believing in me.

Abstract

Adipose tissue is well-recognized as an endocrine organ which secretes a variety of bioactive molecules, including angiotensin II and its precursor angiotensinogen (Agt). There is mounting evidence linking the adipose renin-angiotensin system (RAS) and diet to obesity and obesity-related disorders. However, research addressing dietary regulation and function of adipose RAS is limited, and the specific mechanisms by which PUFAs modulate the endocrine function of adipose tissue remain largely unclear. There are several potential mechanisms that may mediate PUFA effects on Agt, including toll-like receptor signalling, prostaglandins or PPAR-gamma. Thus, we propose to investigate whether PUFAs differentially modulate Agt expression and secretion and to examine possible mechanisms by which PUFA alter Agt expression using the 3T3-L1 cell line.

Differentiated 3T3-L1 adipocytes were treated with arachidonic acid (AA), eicosapentaenoic acid (EPA), AA + EPA, or vehicle (C) for 48 hours. Results showed a significant increase in intracellular Agt protein following treatment with PUFAs. Agt secretion, however, was only increased by AA. Interestingly, there is a dose-dependent decrease in Agt protein levels by EPA suggesting that a minimum concentration of n-3 PUFAs is required to elicit an Agt response. Agt mRNA levels were measured by RT-PCR and results showed a significant increase in Agt mRNA in response to treatment with AA but not EPA. These findings suggest that Agt regulation by PUFAs is complex and occurs both post-transcriptionally and post-translationally.

Changes in mRNA stability may account for the observed effects of PUFAs. Adipocytes were treated with the transcriptional inhibitor actinomycin D (Act D) and Agt mRNA expression was measured over time. Total RNA was also measured at each time point to ensure that Act D treatment was effectively decreasing transcription. Agt mRNA expression was not significantly altered by treatment with EPA while treatment with AA increased Agt mRNA levels. These results suggest that Agt mRNA stability is differentially increased by n-6 but not n-3 PUFAs. Although there are clear effects of AA on Agt secretion and mRNA stability, the signaling pathways mediating this response remain to be determined, and additional studies are necessary to further dissect the underlying mechanisms of this regulation.

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Abbreviations

AA	Arachidonic Acid
ACC	Acetyl CoA Carboxylase
ACE	Angiotensin Converting Enzyme
ACEi	Angiotensin Converting Enzyme Inhibitor
Act D	Actinomycin D
ADD1	Alpha-Adducin 1
Agt	Angiotensinogen
ALA	Alpha-Linolenic Acid
Ang II	Angiotensin II
ANOVA	Analysis of Variance
aP2	Adipocyte Lipid Binding Protein 2
ARB	Angiotensin II Receptor Blocker
AT_1R	Angiotensin Type 1 Receptor
AT_2R	Angiotensin Type 2 Receptor
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complimentary Deoxyribonucleic Acid
C/EBP	CCAAT/Enhancer Binding Protein
CEL	Celecoxib
COX	Cyclooxygenase
$cPLA_2$	Cytosolic Phospholipase A_2
C	Control
DEXA	Dual Energy X-ray Absorptiometry
DGLA	Dihomo-γ-Linolenic Acid
DHA	Docosahexaenoic Acid
DMEM	Dulbecco's Modified Eagle's Media
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EFA	Essential Fatty Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPA	Eicosapentaenoic Acid
EP R	E-Prostanoid Receptor
ERK 1/2	Extracellular Signal-Regulated Kinases 1 and 2
FAS	Fatty Acid Synthase
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GLA	γ-Linolenic Acid
GWA	Genome-Wide Association
HETE	Hydroxyeicosatetraenoic Acid
HPETE	Hydroperoxyeicosatetraenoic Acid
HSL	Hormone Sensitive Lipase
IBMX	1-Methyl-3-Isobutylxanthine
IKKβ	IκB Kinase β

IL-1	Interleukin-1
IL-6	Interleukin-6
IL-6 R	Interleukin-6 Receptor
IRS-1	Insulin Receptor Substrate-1
JNK	c-Jun Amino-Terminal Kinase
LA	Linoleic Acid
LCFA	Long Chain Fatty Acid
LOX	Lipoxygenase
LPL	Lipoprotein Lipase
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
mRNA	Messenger Ribonucleic Acid
MUFA	Monounsaturated Fatty Acid
NF-κB	Nuclear Factor-κB
NSAID	Non-Steroidal Anti-Inflammatory Drugs
OA	Oleic Acid
PAI-1	Plasminogen Activator Inhibitor-1
PAMP	Pathogen-Associated Molecular Pattern
PG	Prostaglandin
PGI	Prostacyclin
PI3K	Phosphoinositide-3 Kinase
PPAR	Peroxisome Proliferator Activated Receptor
PRR	Pattern Recognition Receptor
P/S	Penicillin/Streptomycin
PUFA	Polyunsaturated Fatty Acid
LRR	Leucine-Rich Repeat
RAS	Renin-Angiotensin System
RIPA	Radio-Immunoprecipitation Assay
RNA	Ribonucleic Acid
RXR	Retinoid X Receptor
RT-PCR	Real-Time Polymerase Chain Reaction
SCD	Stearoyl CoA Desaturase
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SOCS3	Suppressor of Cytokine Signaling 3
SREBP1c	Sterol Regulatory Element Binding Protein 1c
STAT3	Signal Transducer and Activator of Transcription 3
SVF	Stromal Vascular Fraction
T2DM	Type II Diabetes Mellitus
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline with Tween
TIR	Toll/Interleukin-1 Receptor
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor-α
WHO	World Health Organization
WHR	Waist-to-Hip Circumference Ratio

Chapter 1: Introduction

Obesity has become a worldwide epidemic. The World Health Organization (WHO) reported in 2005 that globally, at least 400 million adults are considered to be obese, and the number of overweight or obese children has more than tripled in the last 20 years (59). Excess body mass is a major risk factor for developing number of diseases, including type II diabetes mellitus, hypertension, and cardiovascular disease, which is the leading cause of death worldwide.

Adipose tissue is traditionally thought of solely as the major depot for energy storage. However, it is now well established that this tissue also acts as an endocrine organ which secretes bioactive peptides called adipokines (3). An imbalance of these peptides contributes to obesity and the development of obesity-related disorders via paracrine, autocrine, and endocrine effects (120). Of specific interest to our lab is the hypertensive hormone Ang II and its precursor Agt. Ang II secreted from adipocytes has been shown to contribute to circulating plasma Ang II, which functions in blood pressure regulation (96). Pharmacological blockade of RAS by angiotensin II receptor blockers (ARB) or ACE inhibitors (ACEi) reduces the anti-adipogenic effects of Ang II in adipose tissue and prevents diet-induced obesity and insulin resistance (85). Consequently, Ang II is implicated as being a potentially significant factor in the development of obesity-related hypertension in conditions of expanded adipose mass.

Research shows that excessive consumption of saturated fat negatively impacts several biomarkers of health. In contrast, polyunsaturated fatty acids (PUFAs), especially omega-3 (n-3) PUFAs are considered to be beneficial to human health by reducing adiposity, repressing lipogenesis and promoting beta-oxidation of fatty acids, as well as by suppressing lipogenic gene expression in adipocytes (39, 65, 88, 112). Thus, diets rich in PUFAs are able to alter adipose tissue gene expression and metabolism.

There is mounting evidence linking adipose RAS and diet to obesity and obesity-related disorders. Indeed, studies indicate that Agt is a fatty acid-responsive gene, and PUFAs increase Agt expression in a differentiation-dependent manner (110). Further, n-3 and n-6 PUFAs were shown to modulate adipogenic and lipogenic gene expression in rodent models (39, 65, 88). In addition, Agt expression is altered in obese versus lean subjects, with most obese animal models

having higher expression versus lean counterparts (49, 56, 143). The regulation of gene expression in adipose tissue may contribute to the beneficial effects of n-3 PUFAs. PUFAs may alter RAS expression and activity to improve blood pressure and insulin action, and decrease inflammation. Although studies clearly show that adipose Agt is nutritionally regulated in parallel with changes in adipogenesis and lipogenesis, it is not known which mechanisms govern the regulation of Agt gene or protein expression by PUFAs (n-3 and n-6) versus saturated or monounsaturated fatty acids. Further, there are several potential mechanisms for fatty acid regulation of Agt in adipocytes, including toll-like receptors (TLRs), peroxisome proliferator activated receptors (PPARs) or prostaglandins (PGs).

Obesity is associated with chronic low-grade inflammation characterized by an increase in inflammatory cytokines and adipokines. There is a considerable amount of data linking proinflammatory cytokine signaling pathways, specifically the toll-like receptor 4 (TLR4) pathway, to the development of insulin resistance and type 2 diabetes (T2D) in conditions of expanded adipose mass (124). Further, saturated fatty acids can activate toll-like receptor 4 (TLR4) and TLR2 signaling in adipocytes leading to increased transcription of inflammatory markers via NF-κB. The prevalence of obesity and the implication of dietary fat in the development of obesity and obesity-related diseases necessitate a better understanding of the mechanisms by which specific nutrients influence fat cell function. While data support a relationship between adipose RAS, inflammation, obesity and diet, the specific mechanisms by which PUFAs modulate the endocrine function of adipose tissue are largely unclear. Thus, we propose to investigate whether PUFAs alter Agt expression in adipocytes and the mechanisms by which this regulation occurs. Elucidation of the mechanisms regulating RAS in adipocytes will provide a better understanding of the role of dietary fat in the development of obesity as well as the mechanisms through which specific nutrients influence fat cell function. We hypothesize that n-3 PUFAs differentially regulate adipose RAS expression and positively alter adipocyte metabolism, and that mechanisms involved include altered TLRs and/or prostaglandins. Specifically, our objectives are

1. To determine if PUFAs differentially regulate Agt expression and secretion
2. To investigate mechanisms of regulation of Agt by PUFAs

Chapter 2: Literature Review

I. Obesity

A. Incidence and Causes

Obesity has become a worldwide epidemic. More than 1.7 billion people globally are classified overweight or obese and it has been estimated that this number will rise to approximately 2.3 billion by 2015 (59). Additionally, the number of overweight or obese children has more than tripled in the last 20 years. Excess body mass is a major risk factor for developing a number of metabolic diseases, including type II diabetes mellitus (T2DM), dyslipidemia, hypertension and cardiovascular disease, which is the leading cause of death worldwide (29, 52, 82).

Weight status is commonly assessed by body mass index (BMI). BMI is calculated as weight in kilograms divided by height in meters squared (**Table 1**). Although an approximation of fat mass is obtained from BMI, it is only an indicator of weight status. The distribution of fat in the body is a significant factor in the development of metabolic disorders associated with obesity. Therefore, direct measurements of body fat can be assessed by skin fold methods or more accurately by dual energy X-ray absorptiometry (DEXA) for diagnosing weight abnormalities and determining body composition.

Table 1. Weight Classifications Based on BMI

BMI	Weight Classification
< 18.5	Underweight
18.5 – 24.9	Normal
25 – 29.9	Overweight
30 – 39.9	Obese
> 40	Morbidly Obese

Obesity is a polygenic disease caused by both gene-gene and gene-environment interactions. Approximately 40 – 60% of the variation in obesity phenotypes such as BMI, fat mass and leptin levels is heritable (94, 95, 104, 131). Extensive research has focused on locating potential susceptibility genes for obesity. Linkage analysis and genome-wide association (GWA) studies are used to identify and map gene variants that confer disease (116, 119, 150). Numerous associations between gene variants and obesity have been reported. Environmental factors have considerable influence on the manifestation of obesity. Indeed, high caloric, unbalanced diet and sedentary lifestyles are significant contributing factors in the development of obesity and related disorders.

B. Adipose Tissue

Adipose tissue is traditionally thought of as the major depot for energy storage. However, it is now well established that this tissue also acts as an endocrine organ that secretes bioactive peptides called adipokines (3). Experimental data have demonstrated that adipokines are important regulators of whole-body homeostasis, and an imbalance of these peptides contributes to obesity and the development of obesity-related disorders via paracrine, autocrine, and endocrine effects (1, 68, 109).

Adipose tissue is made up of a highly heterogeneous mixture of cells. In addition to adipocytes, adipose tissue also contains preadipocytes, pericytes, monocytes, macrophages, stem cells, endothelial cells and vascular smooth muscle cells, which are collectively called the stromal vascular fraction (SVF) (30). The increase in adipose tissue cytokine expression coupled with changes in adipose tissue metabolism and endocrine function contribute significantly to the pathogenesis of the metabolic syndrome associated with obesity.

Excess energy intake increases fat deposition in white adipose tissue. Both adipocyte size and number increase in parallel with expanding adipose tissue mass (12). Adipocyte hypertrophy and preadipocyte hyperplasia are associated with abnormalities in adipocyte metabolism and function leading to an increase in the production and secretion of hormones and proteins from adipose tissue. Indeed, excessive fat mass is positively correlated with the expression of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and

interleukin-6 (IL-6). Adipose tissue produces and secretes cytokines and hormones that act via endocrine effects to influence insulin sensitivity and inflammatory status. Regulation of cytokine secretion from adipocytes is vital for proper glucose and fat metabolism to occur. Increased expression of cytokines in adipose tissue decreases insulin sensitivity and increases lipolysis (64).

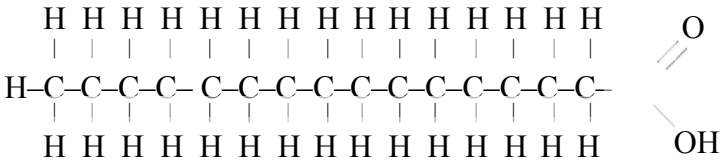
Visceral fat has been shown to have a greater contribution to the metabolic syndrome compared to subcutaneous fat (6, 33, 45). Visceral fat secretion of adipokines associated with systemic inflammation in obese humans (43). Proinflammatory molecules are decreased upon weight loss while expression of anti-inflammatory molecules increases (23). The expression of many adipokines is also fat depot-dependent (7, 32). Abnormal regulation of cytokine production by adipocytes associated with visceral fat accumulation appears to cause dyslipidemia, hypertension, and glucose intolerance in metabolic syndrome (29). This effect is specific to visceral fat and is not observed in subcutaneous adipose tissue. A significant positive correlation between mRNA and protein expression and waist-to-hip circumference ratio has also been reported in visceral fat (144). Thus, it is evident that excess visceral adipose tissue negatively impacts whole-body physiology on multiple levels.

II. Polyunsaturated Fatty Acids

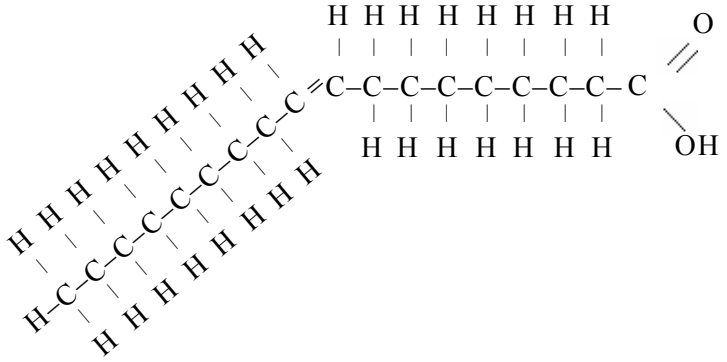
A. Structure and Metabolism

Fatty acids are comprised of a carboxylate head group at the alpha end and long hydrocarbon chains of varying lengths (**Figure 1**). Classification of fatty acids is based on the number of carbons comprising the hydrocarbon chain as well as the degree of unsaturation, or number of double bonds, and the position of these double bonds. Saturated fatty acids lack double bonds and are typically linear in structure allowing them to pack together densely. Monounsaturated fatty acids (MUFAs) contain one double bond and polyunsaturated fatty acids (PUFAs) contain two or more double bonds. Classification of fatty acids is based on the number and position of double bonds. EPA has three carbon atoms separating the last double bond from the terminal methyl (CH_3), or the ω carbon, whereas AA has six carbon atoms separating the last double bond from the ω carbon.

Membrane fluidity is largely determined by the fatty acid composition of the phospholipid bilayer. Unsaturated fatty acids have a lower melting temperature than saturated fatty acids. The presence of double bonds causes bending of the hydrocarbon chain into either the cis or trans configuration. Fatty acids cannot pack together and there is a reduction in the strength of intermolecular forces between fatty acids. This lowers the melting temperature making the bilayer more fluid. By altering membrane fluidity, fatty acids can directly affect cellular activity. Changes in the fatty acid composition of membrane phospholipids influence the structure and fluidity of cell membranes which in turn could alter insulin signaling and eicosanoid biosynthesis pathways. Indeed, studies show that fatty acid composition is associated with insulin resistance (134, 145). Saturated fatty acids impair insulin signaling pathways necessary for glucose uptake resulting in the development of insulin resistance.



Palmitic Acid (16:0), saturated fatty acid



Oleic Acid (18:1 Δ^9), monounsaturated fatty acid

Figure 1: Structures of Saturated and Unsaturated Fatty Acids

The majority of the fatty acids are synthesized in the body. Essential fatty acids (EFAs), however, must be obtained from the diet. There are two classes of EFAs: omega-6 and omega-3 PUFAs. Dietary linoleic acid (LA) is converted into arachidonic acid by a series of desaturation and elongation steps catalyzed by the enzymes Δ^6- and Δ^5-desaturases (desaturation), and elongase (elongation) (**Figure 2**). Similarly, dietary α-linolenic acid is converted into eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EFAs produce hormones that regulate numerous metabolic processes such as inflammation and cholesterol metabolism, and supply the body with energy for cell function.

B. Eicosanoid Biosynthesis

Eicosanoids are generated from the oxidation of LA and ALA. They are functionally diverse and are invovled in numerous processes such as regulation of blood pressure, inflammation, immune system modulation, blood clotting, regulation of sleep/wake cycles, and control of reproductive processes and tissue growth (129).The four major families of eicosanoids include prostaglandins, prostacyclins, thromboxanes and leukotrienes.

EFAs must be liberated from the membrane in order to synthesize eicosanoids. Accordingly, AA and EPA are released from the membrane phospholipids by phospholipase A_2 ($cPLA_2$). $cPLA_2$ catalyzes the hydrolysis of the ester linkage between the fatty acid and the glycerol backbone of the lipid bilayer. This is the rate-limiting step for eicosanoid biosynthesis (80). Free AA is converted into 2-series prostaglandins (PGE_2) and free EPA is converted into 3-series prostaglandins (PGE_3). Cyclooxygenase (COX) catalyzes the oxygenation of AA or EPA into prostanoids (prostaglandins, prostacyclins and thromboxanes). Similarly, lipoxygenase (LOX) catalyzes the synthesis of leukotrienes. Eicosanoids generated from EPA decrease inflammation whereas those from AA promote a proinflammatory state. Because EPA is structurally similar to AA (**Figure 3**), it can compete with AA for incorporation into membrane phospholipids and become the preferential substrate for COX. Oxygenation of AA by COX is competitively inhibited by n-3 PUFAs and PGE_3 is generated instead of PGE_2. Indeed, treatment with pre-formed EPA in the diet decreases PGE_2 levels through competitive inhibition of AA (81). Thus, PGE_2 levels can be manipulated by increasing consumption of EPA.

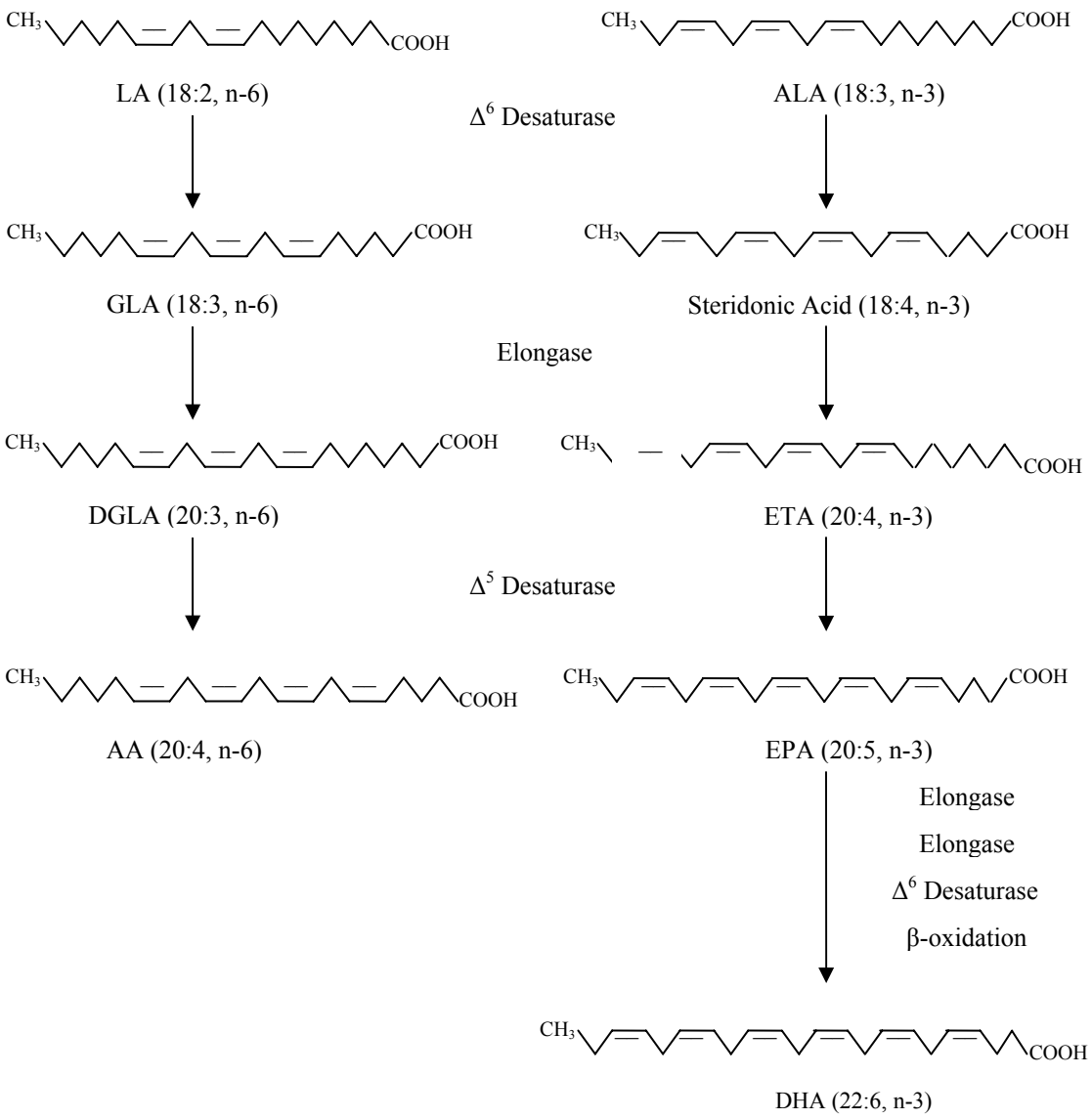
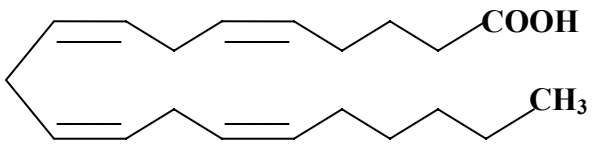
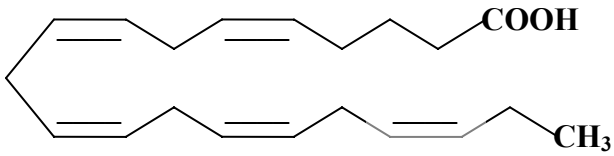


Figure 2: Generation of AA and EPA from Essential Fatty Acids

Linoleic acid (LA) and α-linolenic acid (ALA) undergo a series of desaturation and elongation steps to generate arachidonic acid (AA) and eicosapentaenoic acid (EPA), respectively. Desaturation of LA is first catalyzed by Δ^6 desaturase to generate γ-linolenic acid (GLA), which is then elongated via elongase to form dihomo-γ-linolenic acid (DGLA). Desaturation of DGLA is catalyzed by Δ^5 desaturase to yield AA. Similarly, ALA is desaturated first by Δ^6 desaturase to steridonic acid, elongated via elongase to form eicosatraenoic acid (ETA), and then desaturated again by Δ^5 desaturase to generate EPA. Further desaturation of EPA produces docosahexaenoic acid (DHA).



Arachidonic Acid (20:4, n-6)



Eicosapentaenoic Acid (20:5, n-3)

Figure 3: Structures of AA and EPA

The chemical structures of AA (n-6) and EPA (n-3) are similar and differ only by the presence of an extra double bond in EPA. Structural similarities allow EPA to compete with AA for binding to the COX enzyme.

COX, also known as prostaglandin H_2 synthase (PGH_2 synthase), has both cyclooxygenase and peroxidase activity. The COX complex is made up of COX-1 and COX-2 enzymes. COX-1 is constitutively expressed while COX-2 expression is inducible and highly regulated. Pharmacological inhibition of COX-2 with celecoxib (CEL) decreases PGE_2 secretion in adipocytes, subsequently decreasing lipolysis (148). Corticosteroids are inhibitors of $cPLA_2$ expression thus reducing the amount of AA that is released from the membrane (**Figure 4**). Non-steroidal anti-inflammatory drugs (NSAIDs) like aspirin and ibuprofen non-selectively inhibit both COX-1 and COX-2 activity. Aspirin binds irreversibly to the active site of COX preventing AA from binding.

C. PUFAs and Obesity

Research shows that excessive consumption of saturated fat negatively impacts several biomarkers of health. Obesity and metabolic syndrome are positively correlated with saturated

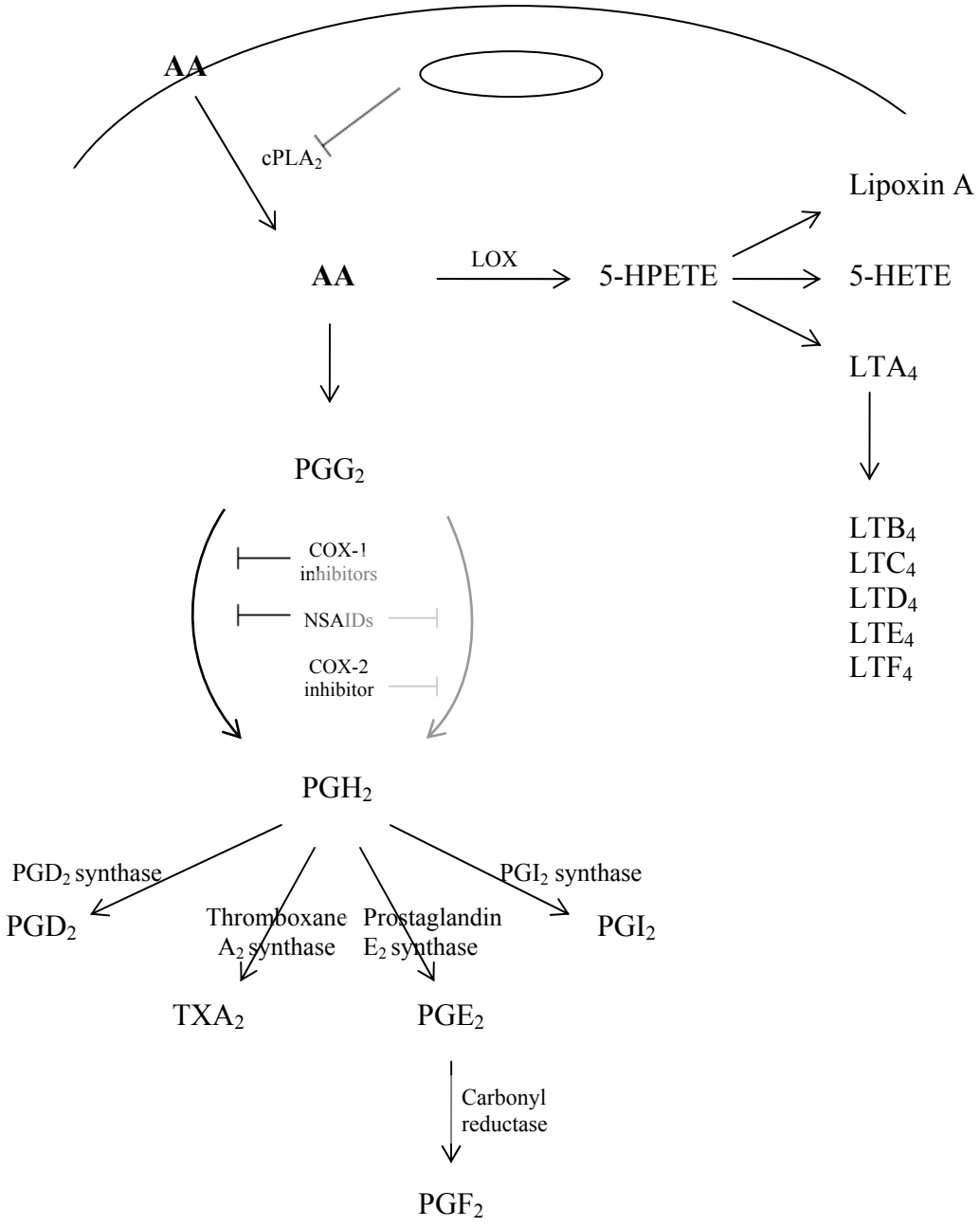


Figure 4: Eicosanoid Biosynthesis Pathways and Inhibitors

AA is liberated from the membrane by cytosolic phospholipase A_2 ($cPLA_2$), converted to PGG_2 and PGH_2 by cyclooxygenase (COX), and then converted by specific synthases to 2-series prostaglandins (PG), prostacyclins (PGI), and thromboxanes (TX). Non-steroidal anti-inflammatory drugs (NSAIDs) and isoform specific COX inhibitors block eicosanoids production. AA is also converted to hydroxyeicosatetraenoic acid (5-HETE), hydroperoxyeicosatetraenoic acid (5-HPETE), and leukotrienes (LT) via lipoxygenase (LOX).

fatty acid intake (4, 58, 128). However, PUFAs, especially those from the n-3 family, are considered to be beneficial to human health by reducing adiposity, repressing lipogenesis, and promoting β-oxidation of fatty acids, as well as suppressing lipogenic gene expression in adipocytes (39, 65, 88, 112).

PUFAs can alter metabolic processes such as carbohydrate and lipid metabolism. The n-3 family, and to an extent the n-6 family of PUFAs, inhibit lipogenic gene transcription in the liver as well as in adipose tissue, promoting β-oxidation of fatty acids and repressing lipid synthesis and storage (6, 67, 112). Thus, there is a shift from lipid synthesis and storage to lipid oxidation. Dietary n-3 PUFAs act as ligand activators of PPARα, which induces the transcription of genes invovled in β-oxidation (99). The effects of PUFAs on lipogenic gene expression in adipose tissue have also been examined. It was found that PUFAs inhibit transcription of lipogenic genes such as fatty acid synthase (FAS), lipoprotein lipase (LPL), hormone-sensitive lipase (HSL), adipocyte lipid-binding protein (aP2), and acetyl-CoA carboxylase (ACC), and down-regulate the enzyme stearoyl-CoA desaturase-1 (SCD1), which catalyzes and regulates the biosynthesis of monounsaturated fatty acids and whose regulation parallels that of lipogenic enzymes (67, 121).

The regulation of the enzyme SCD1 is physiologically important, and changes in SCD1 activity affect a number of physiological variables such as adiposity, atherosclerosis, and obesity (140). Inhibitory effects by PUFAs on the expression of SCD1 have been reported in cultured murine adipocytes (67, 121). These effects may play a central role in the repression of lipogenesis. SCD1 catalyzes the first regulatory step in the biosynthesis of monounsaturated fatty acids, which are central components of membrane phospholipids, cholesterol esters, and triglycerides. PUFAs suppress the production and accumulation of monounsaturated n-9 fatty acids, such as oleic acid, by inhibiting SCD1 gene expression (67, 122). Our lab previously demonstrated that PUFAs decrease SCD1 mRNA levels in adipose tissue of both lean and obese Zucker rats. In addition, we showed that there is a dose-dependent down-regulation of SCD1 expression by PUFAs in mouse 3T3-L1 adipocytes (67). Other studies have reported similar results using linoleic acid, arachidonic acid, and eicosapentaenoic acid (121). Thus, it is apparent that diets rich in PUFAs are able to positively alter adipose tissue gene expression and metabolism.

III. Renin-Angiotensin System

A. Systemic/Classic RAS

Classically, the renin-angiotensin system (RAS) is an important regulator of systemic blood flow and fluid and electrolyte homeostasis. Changes in blood volume stimulate secretion of the RAS components from various tissues into the bloodstream. Circulating angiotensin II (Ang II) is the main effector of the renin-angiotensin system (RAS). It is generated from two enzymatic cleavages of the 452-amino acid precursor angiotensinogen (Agt) by renin and angiotensin converting enzyme (ACE) (13) (**Figure 5**). Agt and Ang I can also be cleaved by a number of other enzymes, including proteases aminopeptidases, carboxypeptidases and endopeptidases (118). The conversion of Agt to Ang I is the rate-limiting step and an increase in Agt results in an increase in Ang II synthesis. Ang II acts through the angiotensin type 1 receptor

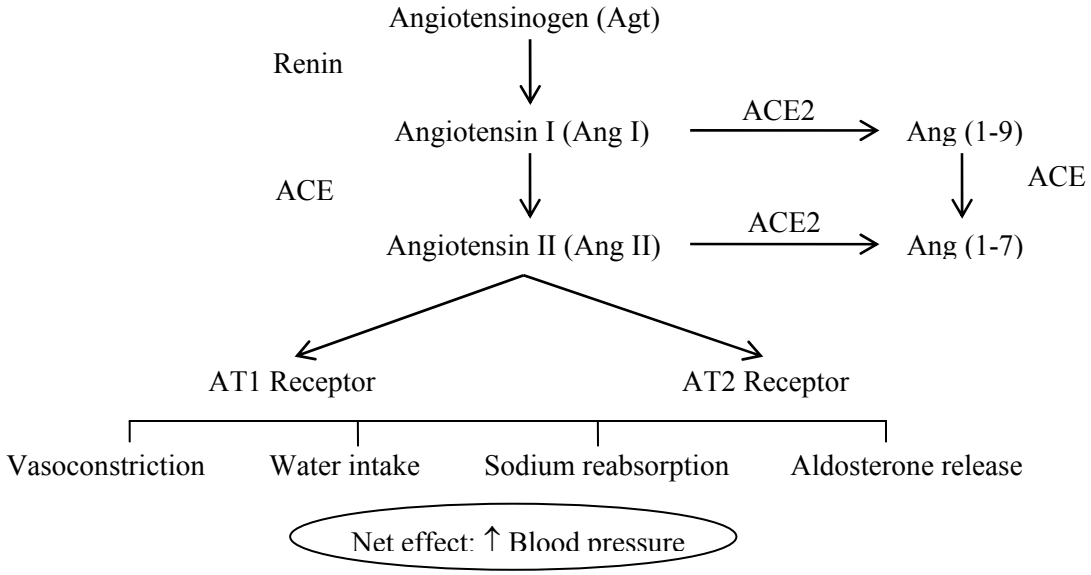


Figure 5: The Renin-Angiotensin System

Angiotensinogen (Agt) is cleaved twice, first by renin into angiotensin I (Ang I) and then by angiotensin converting enzyme (ACE) into angiotensin II (Ang II) to activate RAS. Ang II then binds to angiotensin type 1 (AT1) or 2 (AT2) receptors. Ang (1-9) and Ang (1-7) are also produced by ACE2-mediated cleavage of Ang I or Ang II, respectively.

(AT_1R) and angiotensin type II receptor (AT_2R) at the cell membrane to exert paracrine and endocrine effects.

Other biologically active angiotensin peptides have also been identified, including Ang (1-7) and Ang (1-9) (**Table 2**). ACE2, a homolog of the ACE enzyme, is the newest component of the RAS (31, 139). The carboxypeptidase activity of ACE2 produces Ang (1-7) and Ang (1-9) from Ang II and Ang I, respectively (114) although catalytic efficiency is much greater for Ang II than Ang I (146). Ang (1-7) opposes the activity of Ang II to regulate blood pressure (40, 41). Indeed, deletion of ACE2 in mice results in a modest increase in blood pressure and an elevated response to Ang II (55). Ang (1-7) also has protective effects on cardiac funtion by preventing cardiac remodeling (42, 115).

The components of the systemic RAS are produced and secreted from different organs into the circulation where they interact to form bioactive Ang II. Agt is dervied from the liver, ACE from the vasculature, and renin from the kidneys. However, recent studies have demonstrated that RAS expression is not limited to the circulation. The RAS components are also expressed locally in a variety of other tissues, including the brain, heart, prostate, pancreas, and adipose tissue. The existence of a complete and functional RAS within a tissue or organ suggests that local RAS may play an important role in whole-body physiology (35, 69, 86).

Table 2. Amino Acid Sequences of Angiotensin Peptides

Angiotensin Peptide	Amino Acid Sequence
Agt	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser
Ang I	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
Ang II	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
Ang (2-8) (Ang III)	Arg-Val-Tyr-Ile-His-Pro-Phe
Ang (1-7)	Asp-Arg-Val-Tyr-Ile-His-Pro
Ang (3-7)	Val-Tyr-Ile-His-Pro
Ang (3-8) (Ang IV)	Val-Tyr-Ile-His-Pro-Phe
Ang (1-9)	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His
Ang (1-12)	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr

B. Local RAS

Agt secreted from adipose tissue exerts both paracrine and autocrine effects within adipose tissue. This hormone modulates adipocyte metabolism by increasing adipocyte fatty acid and triglyceride synthesis via receptor-mediated paracrine effects (28, 68, 75). Our laboratory demonstrated that mouse, rat and human adipose tissue express several components of the renin-angiotensin system, including Agt mRNA and protein (69). This gene was differentially expressed in lean versus obese A^{vy}/a mice and Zucker rats. In addition, Ang II secreted by adipose tissue regulates adipocyte metabolism acts via paracrine effects. Ang II induces the production and secretion of prostacyclin (PGI_2), which stimulates lipogenic gene transcription in a glucose-dependent manner leading to adipocyte differentiation (28, 68). Supportive evidence has been published by our laboratory demonstrating that the treatment of human adipocytes with Ang II increases fat synthesis and triglyceride stores (68, 74, 75). Additionally, we have shown that leptin and FAS gene transcription are up-regulated by Ang II in human adipocytes (68). The insulin regulatory element (E box) and the Sterol Regulatory Element Binding Protein 1 (SREBP1) mediated the regulation of FAS by Ang II.

Endocrine effects of adipose Ang II have also been demonstrated using engineered mouse models with targeted inactivation of Agt or with adipose tissue specific expression of Agt (76, 96, 97). These studies provide evidence that adipocyte-derived Agt contributes to circulating Ang II and is involved in kidney and adipose homeostasis. Genetically engineered mouse models with targeted overexpression of Agt in adipose tissue exhibit a significant increase in circulating Agt levels, fat mass and blood pressure compared to wild-type mice (96). Abnormalities in renal structure and function were observed in Agt-deficient mice, as well as altered expression of genes that regulate blood pressure and hypotension. However, these changes could be corrected by re-expressing Agt in adipose tissue, indicating that Agt secreted from adipocytes into the circulation plays a role in blood pressure regulation (76).

It has also been reported that the inactivation of Agt or one of its receptors (AT_1R or AT_2R) prevents high fat diet-induced obesity and insulin resistance in rodents (76, 97, 152). RAS blockade (via receptor antagonism and ACE inhibition) reduces the incidence of type 2 diabetes, improve insulin sensitivity and increase circulating adiponectin levels (25, 46, 77). Genetically

obese rodent models were reported to have much higher Agt expression and secretion after normalizing to cell size compared to lean littermates (56, 69). Agt may also regulate body fat distribution; a significant positive correlation between Agt mRNA expression and waist-to-hip circumference ratio (WHR) was reported in both visceral and subcutaneous adipose tissue in humans (144). Studies have also shown that weight loss reduces Agt expression in adipose tissue in humans.

C. Nutritional Regulation of RAS

Adipose Agt is hormonally and nutritionally regulated in response to changes in adipogenesis and lipogenesis (45, 69). Indeed, adipose Agt has been shown to be regulated by fatty acids and high-fat feeding (34, 53, 110) as well as by Ang II (93). Expression of Agt in murine adipocytes was considerably decreased during fasting and elevated upon refeeding (45). The expression of RAS components in adipose tissue is regulated by fatty acids in a differentiation-dependent manner (28, 110). Agt expression in preadipocytes was shown to be responsive to long-chain fatty acids (LCFAs), which act as transcriptional activators (2). Collectively, these findings suggest that there is a relationship between adipose RAS, obesity and diet. However, differential regulation of Agt by fatty acids (n-3 versus n-6) has not been examined in adipocytes.

IV. Signaling Pathways Related to Fatty Acid Regulation of Gene Expression

A. Insulin Signaling

Insulin is a key hormone in the regulation of glucose metabolism and transport and the binding of insulin to the insulin receptor (IR) activates signal transduction pathways that regulate a number of physiological processes. Circulating insulin stimulates glucose transport into tissues while decreasing gluconeogenesis to regulate blood glucose levels. Insulin also regulates other physiological processes, including lipogenesis, glycogen synthesis, and cell growth and differentiation (21).

The insulin receptor contains an extracellular α-subunit and an intracellular β-subunit. The β-subunit has tyrosine kinase activity and phosphorylates tyrosine residues of insulin receptor substrates (IRS). The binding of insulin to the α-subunit of the insulin receptor stimulates autophosphorylation, which activates the tyrosine kinase activity of the β-subunit. Subsequently, IRS proteins localize to the cell membrane whereupon they undergo tyrosine phosphorylation and activation of signal transduction pathways. Signaling is propagated by the phosphorylation of PI3K leading to GLUT4 translocation and glucose transport, glycogen synthesis, protein synthesis and cell survival (125). IR signaling pathways are regulated by serine phosphorylation of IR or IRS proteins, which inhibits tyrosine phosphorylation and, consequently, the activation of IR signaling pathways.

Insulin resistance often develops as a result of obesity. The effects of fatty acids on insulin signaling has been well-documented (19, 20, 98, 138, 151). Saturated fatty acids as well as n-6 PUFAs decrease tyrosine phosphorylation of IRS-1 (44). Substitution of fish oil into a high-fat diet improves insulin action in rats (127). The renin-angiotensin system also interferes with insulin signaling and action. Indeed, pharmacological inhibition of RAS improves insulin sensitivity (22). Ang II secreted from adipocytes activates phosphoinositide-3 kinase (PI3K)-mediated insulin signaling via receptor-mediated paracrine effects (77).

B. TLRs and NF-κB signaling

Inflammatory pathways of the innate immune system are activated by the recognition of microbial structures called pathogen-associated molecular patterns (PAMPs) by toll-like receptors (TLRs) (66). Currently, 13 families of TLRs have been identified and characterized in mice, 11 of which have also been identified in humans (149). All TLRs are type I transmembrane proteins with a cytoplasmic carboxy-terminal Toll/Interleukin-1 receptor (TIR) domain and an extracellular amino-terminal leucine-rich repeat (LRR) domain. These pattern recognition receptors (PRRs), which are classified by their ligand specificity, are integral to innate immunity; the capacity of the TLRs to respond to the highly diverse PAMPs of viruses, gram-negative and gram-positive bacteria, fungi and parasites (113) enables the eukaryotic host to reliably detect microbial infections. Thus, it is not surprising that the TLRs are ubiquitously expressed on the

surface (TLRs 1, 2, 4, 5 and 6) or in the interior (TLRs 3, 7, 8 and 9) of a variety of immune cells, including monocytes, macrophages, dendritic cells and neutrophils (36) as well as non-immune cells like vascular endothelial cells, lung and intestinal epithelial cells, cardiac myocytes and adipocytes (5). The recognition of PAMPs by their respective TLRs activates the innate immune system.

TLR4 is a pattern recognition receptor which is specifically activated by lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, resulting in increased production of proinflammatory cytokines. TLR4 is expressed in adipocytes and is highly responsive to bacterial LPS in humans (8, 90). Accordingly, human adipocytes contain a high concentration of proinflammatory cytokines (90). The discovery of immune cells, including monocytes, macrophages and leukocytes, in the stromal vascular fraction (SVF) of adipose tissue led to the hypothesis that the majority of the inflammatory responses in adipose tissue are not due to adipocytes but rather these immune cells. Whether adipose tissue macrophages or adipocytes can differentially activate TLR4 has yet to be established. In humans, TLR4 expression and activity in adipocytes is comparable to that of monocytes (8). The response of TLR4 in adipocytes is as sensitive as that of TLR4 in immune cells. The presence of functional TLR4 in adipose tissue and increased production and secretion of proinflammatory cytokines from adipose in conditions of expanded fat mass suggest a potentially significant role of this pathway in the development of obesity-induced inflammation and insulin resistance.

Nuclear factor-kappa B (NF-κB) regulates cellular immune responses to microbial infection by modulating the production proinflammatory cytokines such as IL-2, IL-1 and TNF-α (51, 111). The regulation of immune responses by NF-κB is crucial for preventing chronic activation of proinflammatory pathways. IKKβ is also implicated in the development of insulin resistance. It phosphorylates IκB causing proteasomal degradation, which liberates NF-κB allowing it to translocate into the nucleus and activate the transcription of cytokines. NF-κB is activated by both the mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase/Akt (PI3K/Akt) pathways, and activation promotes the expression of genes whose products cause insulin resistance (126).

V. Proposed Mechanisms for the Regulation of Adipose RAS

Dietary fatty acids are able to alter adipose tissue gene expression and metabolism. PUFAs are beneficial to a number of metabolic processes. A dose-dependent hypotensive effect of n-3 PUFAs was reported in humans (107). They also improve insulin sensitivity and decrease inflammation associated with obesity. Agt and Ang II are also nutritionally regulated. However, research addressing PUFA regulation of RAS in adipose tissue is limited and the mechanisms by which this may occur are unclear.

Several mechanisms may mediate the effects of PUFAs on Agt in adipocytes (**Figure 6**). One possibility is that prostaglandins mediate this regulation. PGE_2 and PGI_2 are produced from AA and have been shown to increased inflammation. They also exert paracrine/autocrine effects on adipocytes (78). Ang II induces the production and secretion of prostacyclin (PGI_2) from mature adipocytes, which stimulates lipogenic gene transcription in a glucose-dependent manner resulting in adipocyte differentiation (28). Low dose PUFAs supplemented in the diets of healthy adults resulted in a decrease in prostaglandin E1 and leukotriene B4 secretion (72, 106). Thus, the effects of PUFAs on Agt in adipocytes may be mediated by changes in prostaglandin levels.

PPARγ plays a central role in regulating adipogenesis. It binds to the promoter and activates the transcription of many lipogenic and adipogenic genes (141). The beneficial effects of n-3 PUFAs on adipogenesis, lipolysis, inflammation and apoptosis could involve the inhibition of PPARγ gene expression (100). However, other studies report that n-3 PUFAs stimulate adipogenesis by interacting with PPARγ (79, 92). PUFAs are able to bind directly to PPARs and modulate the expression and activity of lipogenic or lypolytic genes, and this is a potentially confounding factor in determining the role of PPARs in regulating genes.

Continuous activation of the innate immune system often develops from obesity. Increasing evidence supports a potentially significant role of the innate immune system in the etiology of obesity-related metabolic diseases via inflammatory signaling pathways, specifically the toll-like receptor 4 (TLR4) signaling pathways. TLR signaling leads to the translocation of NF-κB into the nucleus where it increases transcription of proinflammatory cytokines, including IL-6 and TNF- as well as COX-2. IL-6 and TNF-α induce serine phosphorylation of IRS which blocks insulin receptor signaling. The abnormal regulation of cytokine production by adipocytes

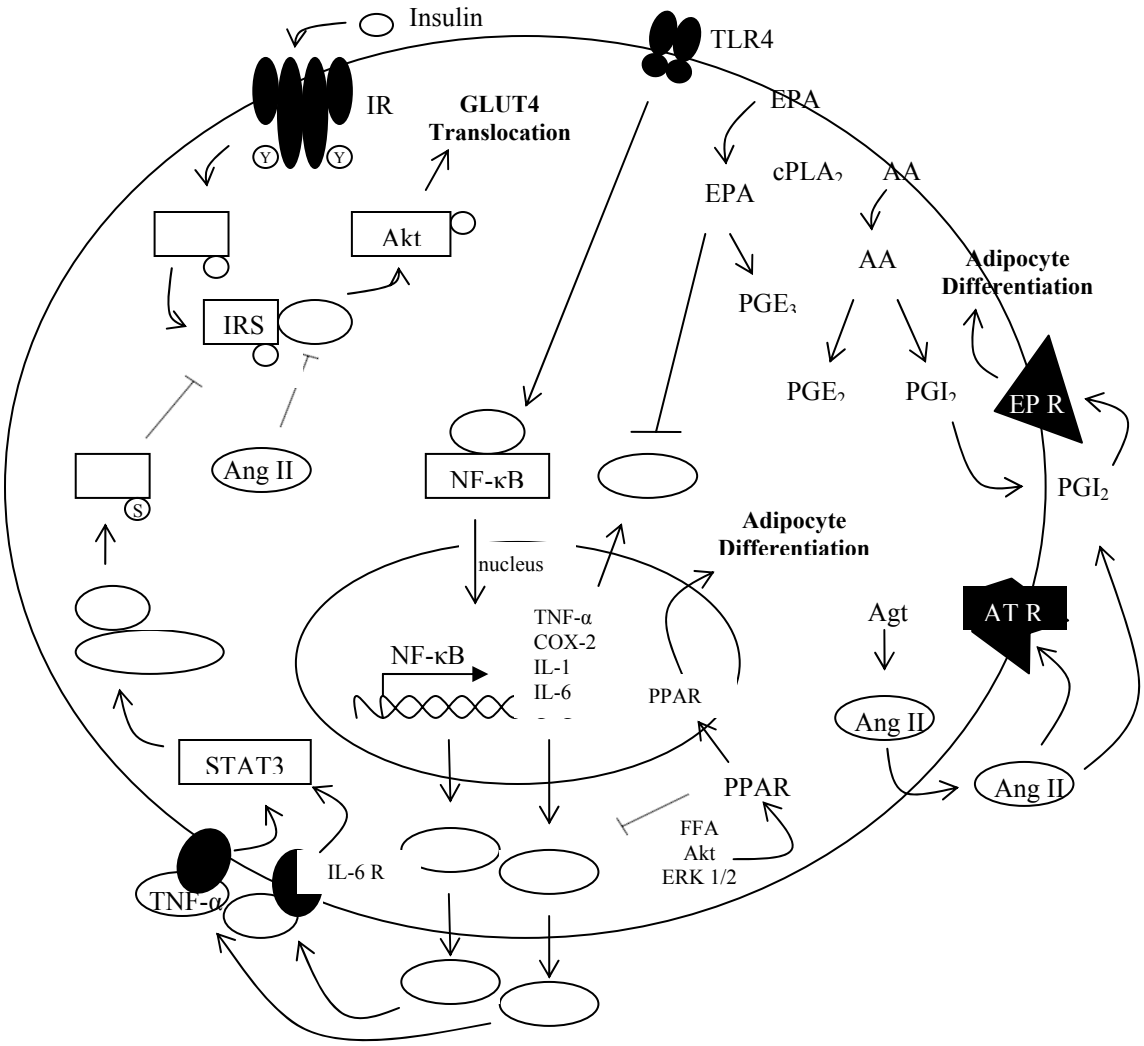


Figure 6: Proposed Mechanisms for the Regulation of Adipose RAS by PUFAs

PGE_2 and PGI_2 are generated from AA and subsequently secreted by adipocytes. They then bind to EP receptors at the surface of the cell and stimulate adipocyte differentiation (PGI_2). EPA competitively inhibits the generation of PGE_2 and PGI_2 from AA to produce PGE_3 and PGI_3. PPARs bind a number of substrates, including FFA, Akt, and ERK 1/2. PUFAs are able to directly bind to PPARs and modulate lipogenic and lypolytic genes. NF-κB is activated by TLR4 signaling and subsequently translocated into the nucleus where it regulates IL-6 and TNF-α gene transcription. IL-6 and TNF-α bind to receptors their respective receptors and activate signaling via STAT3 and JNK and SOCS3 leading to serine phosphorylation of IRS-1 and the disruption of insulin signaling. Ang II secreted from adipocytes regulates fat cell metabolism in part by modulating PI3K-mediated insulin signaling. It also increases PGI2 resulting in increased adipocyte differentiation.

is associated with visceral fat accumulation and appears to cause dyslipidemia, hypertension, and glucose intolerance. Recently, saturated fat was shown to activate TLR4 and TLR2 signaling in adipocytes leading to increased production of proinflammatory cytokines (117). Conversely, n-3 PUFAs have been shown to decrease inflammation by blocking TLR-stimulated upregulation of proinflammatory cytokines (83, 84). Ang II activates many of the same pathways as TLR4. However, it is not know whether TLRs can mediate the effects of PUFAs on Agt expression in adipocytes.

The NF-κB signaling pathway has been implicated in development of obesity-related insulin resistance because it activates the transcription of proinflammatory cytokines that are known to interfere with insulin signaling and action (26). TLR4 signaling activates NF-κB, which is subsequently translocated into the nucleus where it regulates transcription of a number of genes, including TNF-α, IL-6, IL-1 and COX-2. Insulin resistance presumably develops from the disruption of insulin signaling by these proinflammatory cytokines. IL-6 and TNF-α bind to the appropriate receptors and activate signaling. Activated IL-6 and TNF-α induce the phosphorylation of serine residues on IRS-1 thereby disrupting insulin receptor signaling. Whether regulation of Agt by PUFAs is mediated by insulin signaling pathways is not known.

The purpose of this study was to determine whether fatty acids differentially regulate adipocyte Agt expression and secretion in 3T3-L1 adipocytes, and to investigate possible mechanism(s) mediating this regulation. We hypothesize that n-3 PUFAs will differentially modulate adipose RAS expression and positively alter adipocyte metabolism, and that mechanisms involved include altered TLRs and/or prostaglandins.

Chapter 3: Experimental Design and Results

I. Materials and Methods

A. 3T3-L1 Cell Culture

3T3-L1 preadipose cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were cultured in regular growth media consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (P/S). Confluent cells were differentiated into adipocytes by treatment with 0.5 mM 1-methyl-3-isobutylxanthine (IBMX), 0.25 μM dexamethasone and 10 nM insulin (Humulin R®, Eli Lily) for 2 days and then were maintained in regular growth media. Differentiation was considered complete 5-7 days after confluence with ~80-95% of the cells being differentiated. 24 hours prior to treatment, growth media was changed to starvation media consisting of serum-free DMEM with 1% (w/v) fatty acid-free Bovine Serum Albumin (BSA) (Sigma-Aldrich, St. Louis, MO) and 1% P/S.

Cell culture experiments commonly use BSA to bind and stabilize free fatty acids to prevent oxidation as well as to act as a carrier for the fatty acids, which are insoluble in water (18, 54). In addition, albumin binds and transports circulating free fatty acids in vivo making it physiological relevant to use BSA conjugated fatty acids (57). Fatty acids were solubilized in dimethyl sulfoxide (DMSO) and then conjugated to fatty acid-free BSA by agitation in a 37°C water bath for 2 hours prior to treatment. Fatty acid treatment media consisted of serum-free DMEM plus 1% fatty acid-free BSA, 1% P/S, 10 nM insulin and specific treatment (fatty acid or vehicle). Previous dose-response studies indicated that 150 μM AA or EPA (Nu-Check Prep, Inc., Elysian, MN) produced maximal effects on Agt gene expression and Ang II secretion without being toxic to the cell (148). Cells were treated with 150 μM AA, 150 μM EPA, 150 μM AA+EPA (75 μM of each fatty acid), or vehicle (DMSO) for 48 hours.

A dose-response study was previously performed (148). Briefly, 3T3-L1 adipocytes were cultured and differentiated, and EPA and AA were prepared as described above. Differentiated cells were separated into treatment groups and received either 50 μM, 100 μM, 200 μM, or 500

μM of fatty acid (EPA or AA) or vehicle (DMSO). Following 48 hours of treatment, cells were harvested for protein or RNA and stored at -80ºC for future use.

B. mRNA stability assay

Gene expression can be regulated by the stability of the mRNA. The amount of protein that is produced depends on the turnover rate of the corresponding mRNA. Cells were differentiated and treated with fatty acids as described above. Following fatty acid treatment, 1ml of media was collected before the media was aspirated off and then new treatment media was added. The transcriptional inhibitor Actinomycin D (Act D) (Sigma-Aldrich, St. Louis, MO) was added (10 μg/ml) to treated cells as previously reported (24, 27, 70, 133). 1ml of media was collected and cells were harvested in 600 μl of Qiazol lysis buffer at 0, 4, 12 and 24 hours after Act D addition. Total RNA was extracted from these cells and converted into cDNA for subsequent analysis. Agt mRNA abundance was measured by RT-PCR as described below.

C. Western blot analysis

Cells were harvested in 300 μl of radio-immunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail and homogenized for 5 minutes on ice. Protein concentration was measured by Bradford assay using Coomassie G-250 Dye (Thermo Fisher Scientific Inc, Rockford, IL) (11). Normalized protein (30 g) was separated by SDS-PAGE gel electrophoresis at 100 V using a 10% Tris-HCl gel (Bio-Rad, Hercules, CA). Proteins were transferred to a nitrocellulose membrane at 70 V for 3 hours at 4°C with a stir bar. To evaluate transfer efficiency, membranes were stained with Ponceau S (Thermo Fisher Scientific Inc, Rockford, IL). Gels were stained with Coomassie Brilliant Blue R-250 staining solution and destained overnight at 4°C using Coomassie Brilliant Blue R-250 destaining solution (Bio-Rad, Hercules, CA) to verify that protein transfer was successful.

Non-specific binding was blocked using 5% non-fat dry milk (NFDM) in Tris-Buffered Saline (TBS) (Bio-Rad, Hercules, CA) containing 0.1% Tween (TBST) (Fisher, Pittsburgh, PA). Primary antibodies from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA)

included the following: rabbit polyclonal anti-ACE2, anti- AT_1R, anti- AT_2R, anti-GAPDH, anti-PPARγ, anti-PAI-1, anti-phospho-Akt, anti-IRS-1, and anti-vitronectin; goat polyclonal anti-Agt, anti-Renin, anti-TLR2, anti-TLR4, anti-COX-1, and anti-COX-2; and mouse polyclonal anti-Akt. Primary antibodies obtained from Millipore (Billerica, MA) included mouse monoclonal anti-ACE and rabbit polyclonal anti-adiponectin. Horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG and anti-goat IgG secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA).

To visualize proteins, chemiluminescence was performed using SuperSignal West Pico (Thermo Fisher Scientific Inc, Rockford, IL). GAPDH was used as a loading control for cell lysates and vitronectin was used as a loading control for culture media. Relative intensity was quantified by densitometry using ImageQuant TL v2005 software (GE Healthcare Biosciences, Piscataway, NJ). Background was subtracted from the protein of interest (POI) and then band volume intensities were divided by the area to obtain normalized values. The ratio of normalized POI to normalized loading control was calculated to allow for comparisons. Protein expression fold change was determined for each treatment relative to the control.

D. Enzyme-linked immunosorbent assay (ELISA)

ELISAs are a highly sensitive, robust method frequently used to quantitate proteins in culture media or serum (101). It has a wide dynamic range and is able to detect low levels of protein with high sensitivity and minimal little background. Differential expression and secretion of Agt, Ang I, adiponectin, TNF-α and IL-6 in response to treatment with PUFAs was examined by ELISA. Culture media and cells were harvested in RIPA and sonicated as described above. Commercially available ELISA kits for Agt (Immuno-Biological Laboratories Co., Ltd., Minneapolis, MN), Ang I (Phoenix Pharmaceuticals, Inc., Burlingame, CA), adiponectin (Linco Research, Billerica, MA), TNF-α and IL-6 (ALPCO Diagnostics, Salem, NH) were used to assay samples following the manufacturer's protocol. Specific protein concentrations were calculated based on a standard curve generated in each assay.

E. Real-time PCR

The use of real-time polymerase chain reaction (RT-PCR) for mRNA quantitation eliminates the electrophoresis step of traditional Northern blot methods, providing fast, sensitive, and accurate measurements. This methodology has been well-developed and standardized and is commonly used to measure mRNA levels. Gene-specific primers are used to amplify a portion of the gene of interest. A fluorescently-labeled probe binds specifically to the DNA as it is amplified, allowing for continuous quantitation of the amount of amplicon being produced over a period of time. A reporter dye binds new copies of double-stranded DNA. As the amount of amplicon being produced accumulates, the fluorescence intensity increases proportionately. Thus, fluorescence intensity gives an indication of gene expression.

The direct effect of fatty acids on adipocyte mRNA expression were examined in control and fatty acid treated 3T3-L1 cells. Following treatment, cells were harvested in 600 µl of Qiazol lysis reagent (Qiagen, Valencia, CA). Samples were separated by centrifugation at 13000 rpm for 30 mins at 4°C. Total RNA was extracted from adipocytes using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA concentrations and quality and integrity of the RNA were assessed using a NanoDrop (Thermo Fisher Scientific Inc, Rockford, IL). Isolated RNA was then reverse transcribed to cDNA using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Specific mRNA was quantitated from diluted cDNA (1:10) by real-time PCR using the ABI Prism 7000 Sequence Detection System and ABI Prism 7000 SDS software (Applied Biosystems, Carlsbad, California). RT^2 Real-Time SYBR Green (SABiosciences, Frederick, MD) was used as the reporter dye. PCR was performed for 40 cycles. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ comparative threshold cycle method, as previously described (91). β-actin was used as an internal standard to normalize expression data.

Primer sequences for renin, ACE, adiponectin, COX-1, COX-2, PPARγ, TLR1, TLR2, TLR4 and β-actin were obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html) (132, 147) (**Table 3**). Primers were ordered from Sigma Genosys (Sigma-Aldrich, St. Louis, MO). Primers for Agt, AT_1R, AT_2R and FAS were a generous gift from Dr. Brynn Voy (Oak Ridge National Laboratory, Oak Ridge, TN).

Table 3. Primer Sequences for RT-PCR

Gene	PrimerBank ID	Forward Primer (5'→3')	Reverse Primer (5'→3')
ACE	33468873a1	AGGTTGGGCTACTCCAGGAC	GGTGAGTTGTTGTCTGGCTTC
Agt	n/a	CGGAGGCAAATCTGAACAAC	GTCGGCTGTTCCTCCTCTC
AT_1R	n/a	AATGGCTGGCATTTTGTCTG	GCTTTTCTGGGTTGAGTTGG
AT_2R	n/a	GCCTGCATTTTAAGGAGTGC	TGCAGCAACTCCAAATTCTT
β-actin	6671509a3	AGTGTGACGTTGACATCCGTA	GCCAGAGCAGTAATCTCCTTCT
COX-1	6679537a2	CTGTTGTTACTATCCGTGCCAG	CTCAGGGATGGTACAGTTGGG
COX-2	31981525a1	TGAGCAACTATTCCAAACCAGC	GCACGTAGTCTTCGATCACTATC
FAS	n/a	GGAGGTGGTGATAGCCGGTAT	TGGGTAATCCATAGAGCCCAG
PPARγ	6755138a3	TGTGGGGATAAAGCATCAGGC	CCGGCAGTTAAGATCACACCTAT
Renin	13676837a1	CTCTCTGGGCACTCTTGTTGC	GGGAGGTAAGATTGGTCAAGGA
TLR1	13507603a1	TGAGGGTCCTGATAATGTCCTAC	AGAGGTCCAAATGCTTGAGGC
TLR2	31981333a2	ACAATAGAGGGAGACGCCTTT	AGTGTCTGGTAAGGATTTCCCAT
TLR4	10946594a2	GCCTTTCAGGGAATTAAGCTCC	AGATCAACCGATGGACGTGTAA

F. Statistical analysis and data interpretation

Data analysis was performed in SAS 9.2 (SAS Institute, Inc, Cary, NC). Gene expression data and protein fold change values were evaluated for nutrient effects by Student's unpaired t test. Time course data were analyzed using orthogonal polynomial contrasts. Dose response data were analyzed by factorial analysis of variance. Descriptive statistics (mean ± SEM) and comparative statistics were obtained by one-way ANOVA and were used to analyze total RNA. Tukey's test was used for post-hoc analysis when significance was detected. $P < 0.05$ was considered statistically significant for all tests. Data are reported as the mean ± SEM.

II. Results

Effect of PUFAs on Agt expression in 3T3-L1 cells and culture media

Agt protein expression was examined in 3T3-L1 cell lysates and corresponding culture media following treatment with PUFAs. Results from these experiments show a significant increase in intracellular Agt in response to treatment with PUFAs ($P < 0.001$) (**Figure 7**). Agt secretion into the media is consistently increased by treatment with AA ($P < 0.001$) (**Figure 8**).

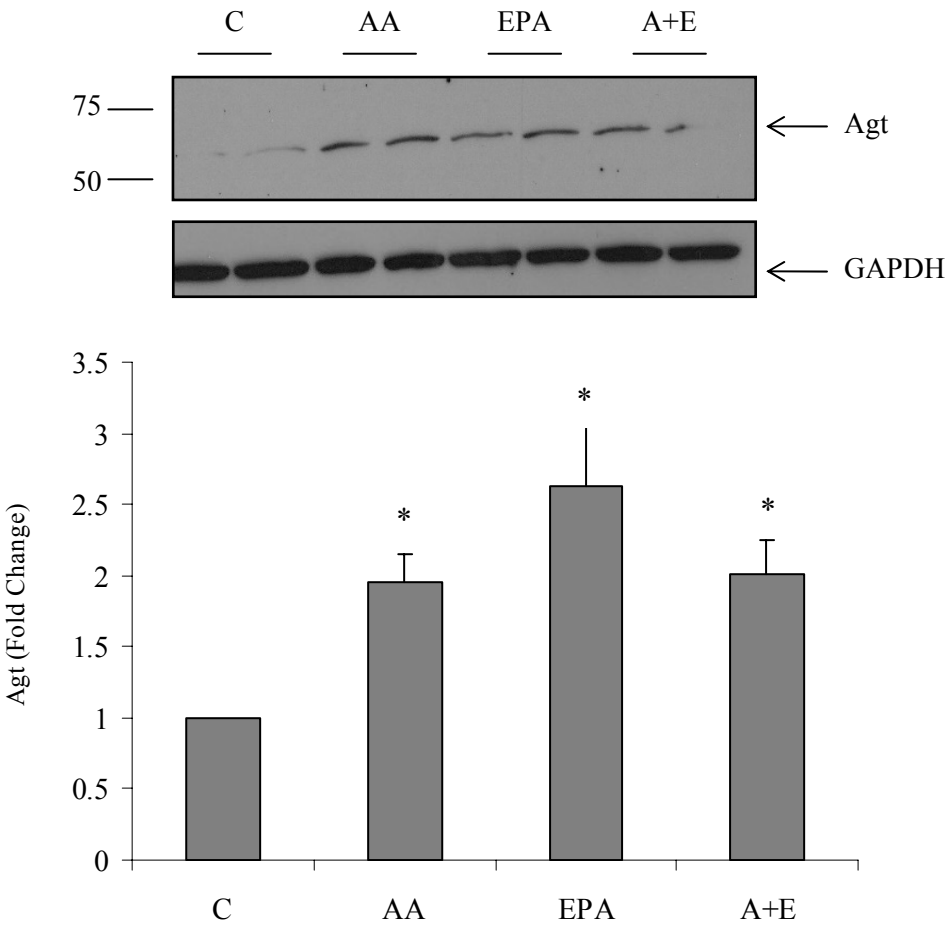


Figure 7: Agt Protein Expression in 3T3-L1 Adipocytes Treated with PUFAs

Western blot is representative of 8 different blots from 3 independent experiments. Lanes 1 and 2, Control (C); Lanes 3 and 4, AA (150 μM); Lanes 5 and 6, EPA (150 μM); Lanes 7 and 8, A+E (150 μM AA+EPA). GAPDH was used as a loading control. The approximate molecular weight of Agt is 60 kD and GAPDH is 37 kD. The graph shows relative fold change of Agt expression in treated cells vs control. n = 16 for all treatments. * $P < 0.001$ vs control.

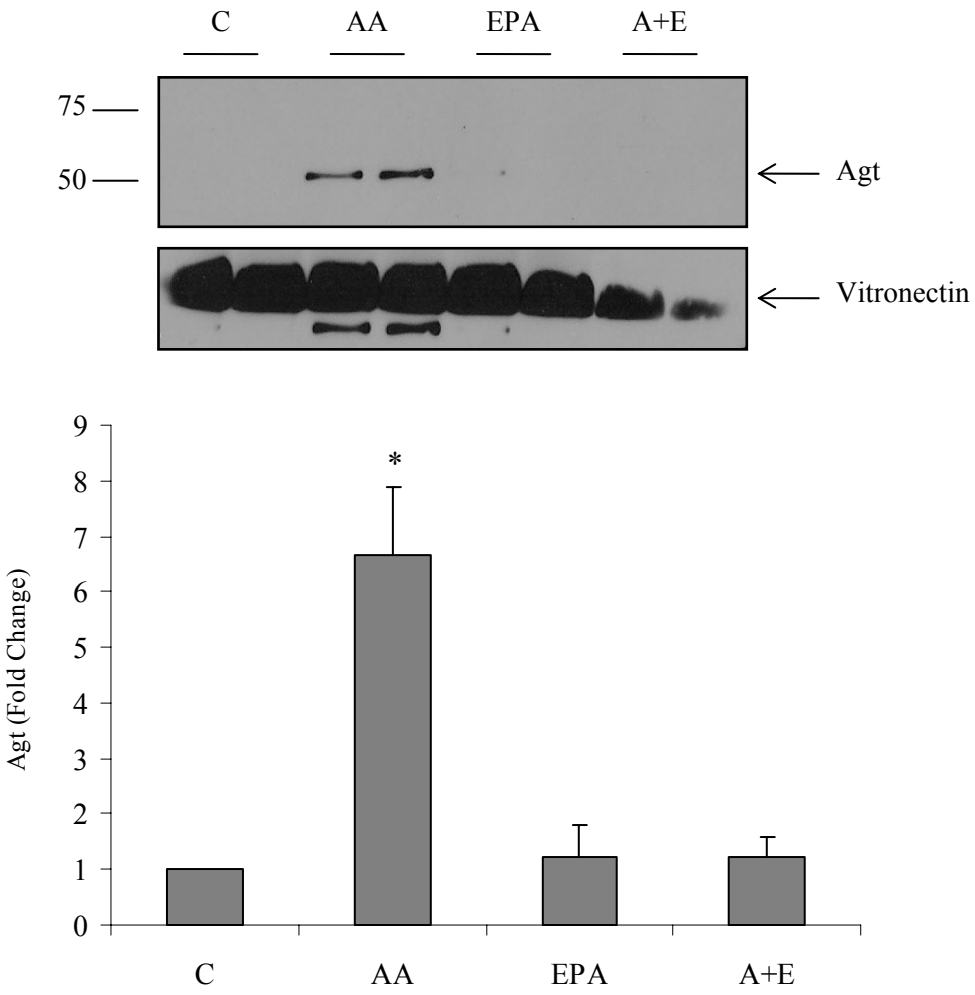


Figure 8: Agt Protein Expression in Culture Media of 3T3-L1 Adipocytes Treated with PUFAs

Western blot is representative of 5 different blots from 3 independent experiments. Lanes 1 and 2, Control (C); Lanes 3 and 4, AA (150 μM); Lanes 5 and 6, EPA (150 μM); Lanes 7 and 8, A+E (150 μM AA+EPA). Vitronectin was used as a loading control. The approximate molecular weight of Agt is 60 kD and vitronectin is 75 kD. The graph shows relative fold change of Agt expression in treated cells vs control. n = 10 for all treatments. * $P < 0.001$ vs control.

This effect was prevented by treatment with EPA. These findings indicate that Agt is differentially regulated by PUFAs and that treatment with n-6 PUFAs increase Agt protein expression and secretion while n-3 PUFAs cause Agt to be retained in the cell. Three repetitions of fatty acid treatments with 150 μM AA, EPA, AA+EPA, or vehicle in 3T3-L1 adipocytes were performed to verify the reproducibility of these results. Indeed, these effects were explicitly reproduced in all three replicate experiments.

mRNA expression levels were also measured following fatty acid treatment to examine the effects of PUFAs on mRNA content. RT-PCR was performed using RNA isolated from 3T3-L1 cells treated with 150 μM AA, EPA, AA+EPA or vehicle. Results show that treatment with AA increases Agt mRNA expression compared to the control ($P < 0.0001$) (**Figure 9**). Conversely, treatment with EPA or combined treatment with AA and EPA did not significantly alter Agt mRNA levels.

A dose response study with PUFAs (AA and EPA) was previously performed by our lab and these samples were used to assess dose effects of PUFAs on Agt protein expression. Differentiated 3T3-L1 cells were treated with 50 μM, 100 μM, 200 μM, or 500 μM of AA or EPA for 48 hours. Agt protein expression was measured in cell lysates and culture media as described in the Materials and Methods section. There was a trend toward increased Agt secretion with increasing doses of AA (**Figure 10**). Interestingly, treatment with increasing doses of EPA appeared to decrease Agt secretion although statistical significance was not reached.

Cell Specific effects of PUFAs on Agt expression

To eliminate the possibility that the effects of PUFAs on Agt expression and secretion are due to preadipocytes, 3T3-L1 preadipocytes were treated with 150 μM AA, 150 μM EPA,150μM AA+EPA or vehicle (DMSO). Agt protein expression was assessed in cell lysates and culture media using differentiated adipocytes treated with vehicle as a positive control. As expected, Agt expression was not detected in preadipocyte cell lysates (**Figure 11**) or culture media (**Figure 12**) but was detected in the positive control sample

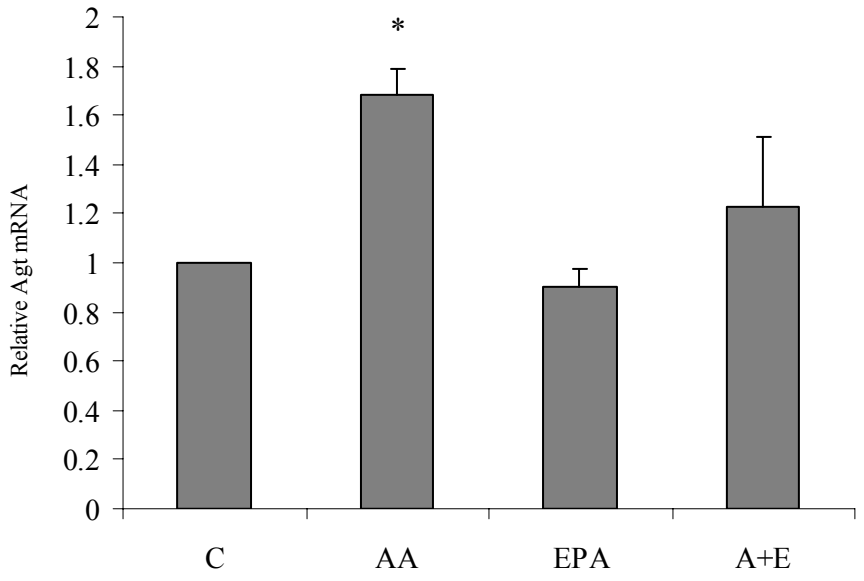


Figure 9: Agt mRNA Expression in 3T3-L1 Adipocytes Treated with PUFAs

Agt mRNA levels were measured in triplicate in adipocytes treated with 150 μM AA, 150 μM EPA, 150 μM AA+EPA (A+E) or vehicle (C). The graph represents the average relative expression values from 3 independent RT-PCR experiments. n = 4 for C, AA and A+E; n = 3 for EPA. * $P < 0.01$ vs control.

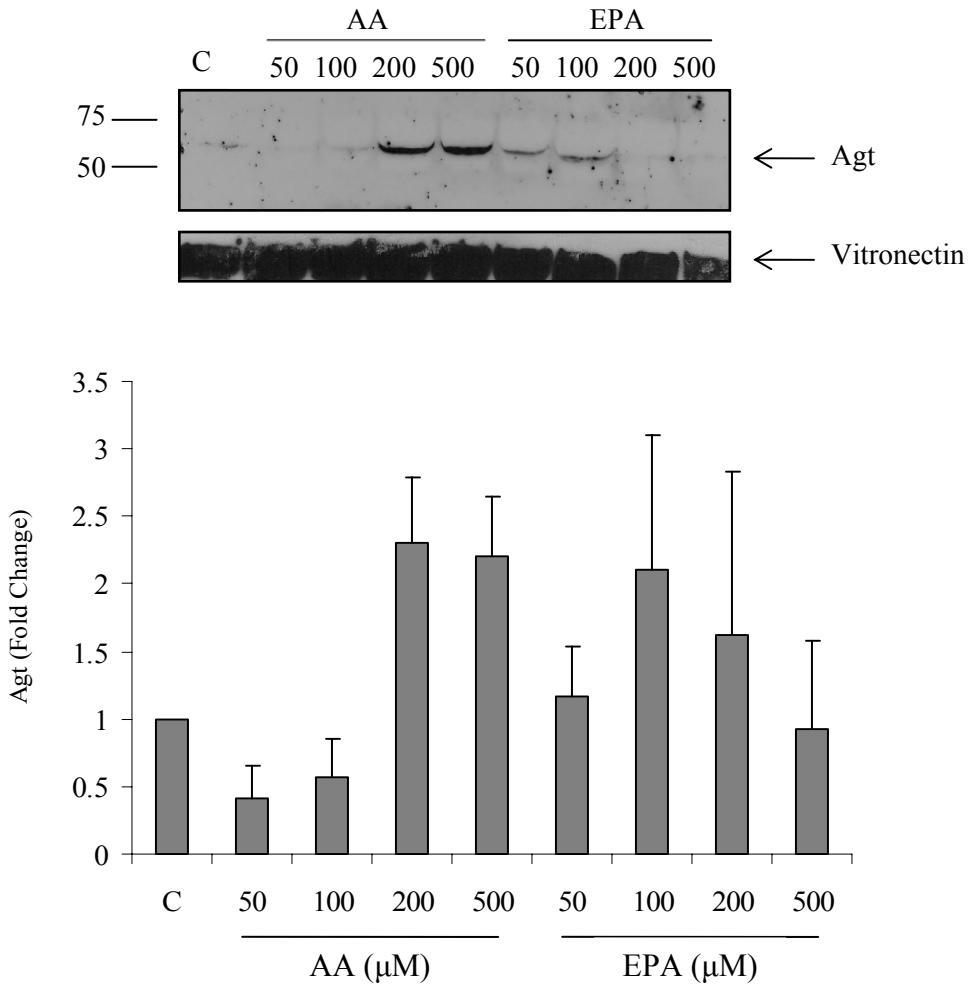


Figure 10: Dose Response Effects of PUFAs on Agt Protein Expression in 3T3-L1 Culture Media

Western blot is representative of 2 different blots. Vitronectin was used as a loading control. The approximate molecular weight of Agt is 60 kD and vitronectin is 75 kD. The graph shows relative fold change of Agt expression in treated cells vs control. n = 2 for all treatments.

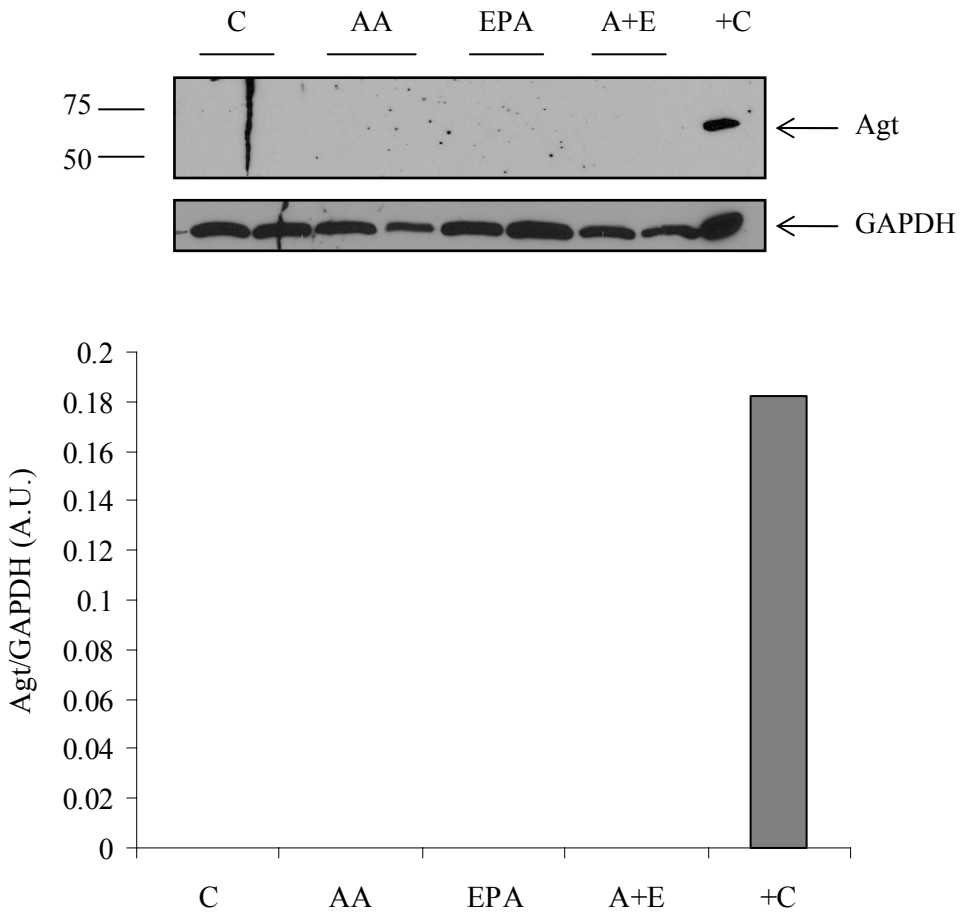


Figure 11: Agt Protein Expression in 3T3-L1 Preadipocytes Treated with PUFAs

Agt expression was assessed in preadipocytes treated with 150 µM AA, 150 µM EPA, 150 µM AA+EPA (A+E) or vehicle (C). Mature adipocytes treated with PUFAs served as a positive control for Agt expression. Lanes 1 and 2, Control (C); Lanes 3 and 4, AA; Lanes 5 and 6, EPA; Lanes 7 and 8, A+E; Lane 9, Positive Control (+C). GAPDH was used as a loading control for cell lysates. The approximate molecular weight of Agt is 60 kD and GAPDH is 37 kD. n = 2 for C, AA, EPA and A+E; n = 1 for +C. A.U., arbitrary units.

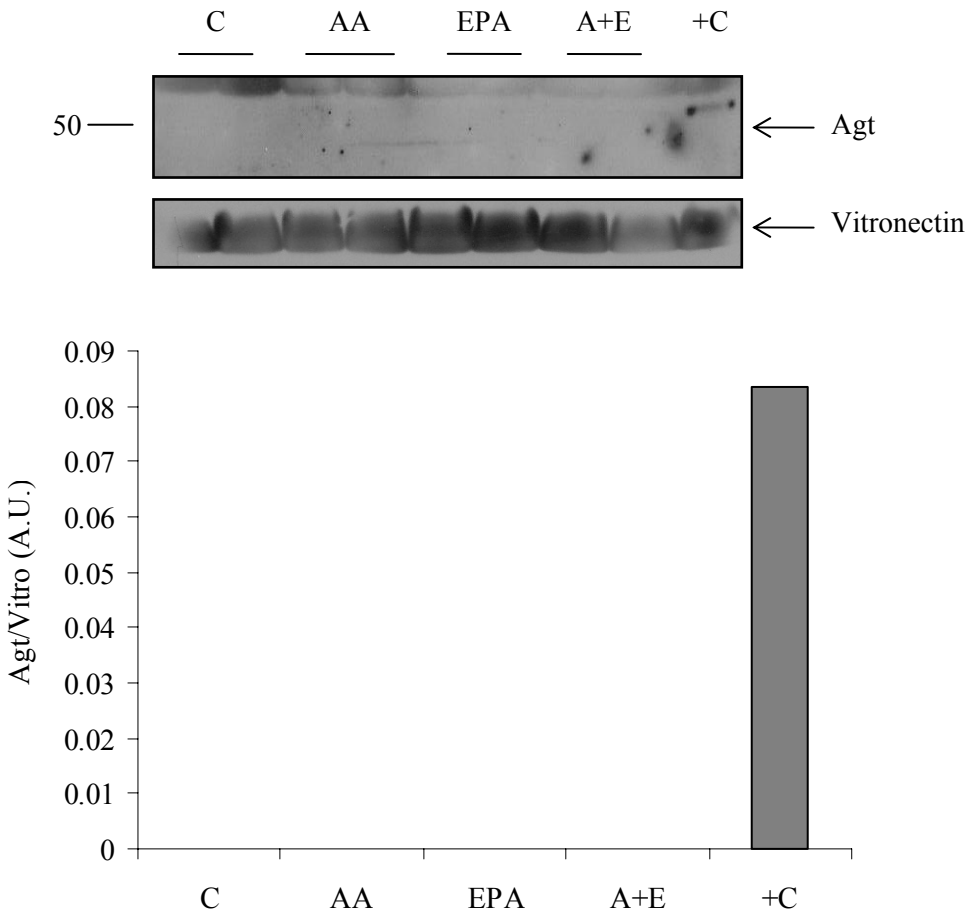


Figure 12: Agt Protein Expression in Culture Media of 3T3-L1 Preadipocyte Treated with PUFAs

Agt expression was assessed in the culture media of preadipocytes treated with 150 µM AA, 150 µM EPA, 150 µM AA+EPA (A+E) or vehicle (C). Mature adipocytes treated with PUFAs served as a positive control for Agt expression. Lanes 1 and 2, Control (C); Lanes 3 and 4, AA (150 µM); Lanes 5 and 6, EPA (150 µM); Lanes 7 and 8, A+E (150 µM AA+EPA); Lane 9, Positive Control (+C). Vitronectin was used as a loading control for media. The approximate molecular weight of Agt is 60 kD and vitronectin is 75 kD. n = 2 for C, AA, EPA and A+E; n = 1 for +C. A.U., arbitrary units; Vitro, vitronectin.

Effect of PUFAs on Ang I levels in 3T3-L1 cells and culture media

The cleavage of Agt into Ang I by the enzyme renin is the rate-limiting step of the RAS pathway. It is possible that the increased secretion of Agt in response to AA is a secondary result of decreased renin activity causing less Agt to be cleaved. Thus, renin activity was indirectly assessed by measuring Ang I levels. Ang I protein levels in the cell and media were measured as an indication of renin activity. Treatment with AA increased Ang I secretion into the culture media ($P < 0.001$) (**Figure 13**). However, Ang I levels were undetectable in cell lysates indicating that levels intracellular levels of Ang I are very low, likely reflecting rapid secretion of Ang I and/or rapid intracellular conversion of the majority of Ang I into Ang II.

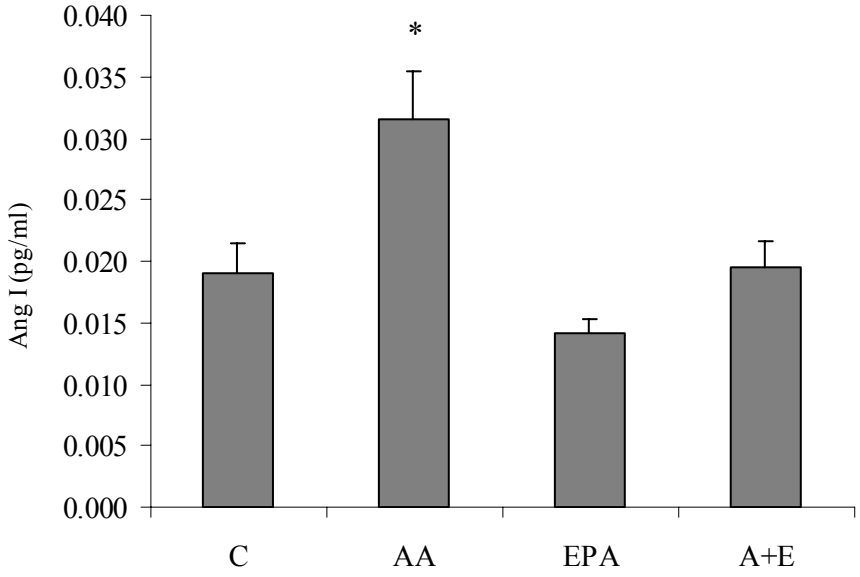


Figure 13: Ang I Protein Levels in Culture Media of 3T3-L1 Adipocytes Treated with PUFAs

Ang I protein levels were measured the culture media treated with 150 μM AA, 150 μM EPA, 150 μM AA+EPA (A+E) or vehicle (C) to assess renin activity in response to PUFAs. n = 8 for all treatments. * $P < 0.001$ vs control.

Effect of PUFAs on Agt mRNA stability and half-life

The stability of mRNA can significanly affect gene expression and subsequently cell growth, differentiation and activity. PUFAs may differentially regulate Agt mRNA stability. Cells were treated with the antibiotic actinomycin D, which inhibits cell proliferation. Total RNA was measured for each time point (0 hours, 4 hours, 12 hours and 24 hours) to assess the degradation of RNA by actinomycin D over time (**Figure 14**). A decrease in total RNA concentration was determined for each of the treatments suggesting that transcription was in fact inhibited by the actinomycin D. To assess the stability of Agt mRNA, Agt mRNA expression levels were also assessed as a function of time (**Figure 15**). Agt levels were determined as a percentage prior to the addition of actinomycin D. Treatment with EPA did not significantly alter Agt mRNA levels. Treatment with AA, however, significantly increased Agt mRNA levels at 4 hours ($P < 0.0001$) and 24 hours ($P < 0.05$).

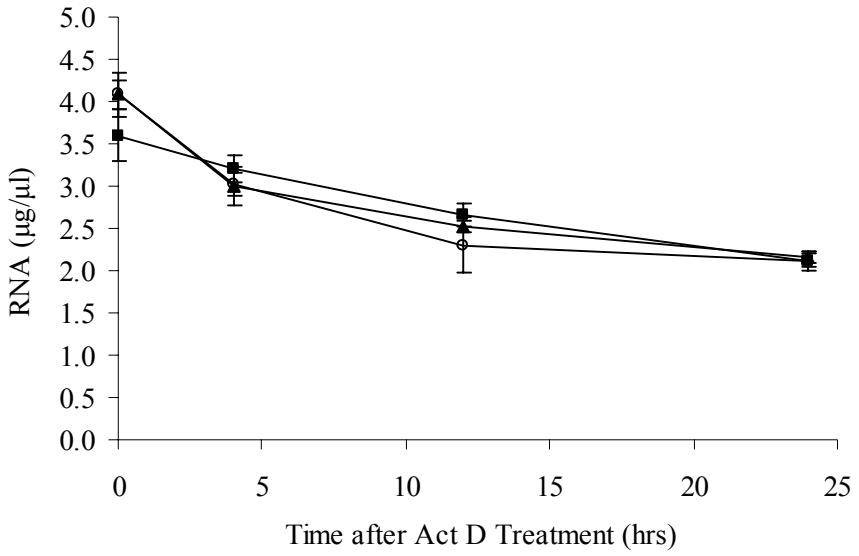


Figure 14: Time Course Analysis of Total RNA in 3T3-L1 Adipocytes Treated with PUFAs

Total RNA was measured in adipocytes treated with PUFAs. The graph represents total RNA levels at 0 hrs, 4 hrs, 12 hrs and 24 hrs following the treatment of actinomycin D (10 µg/ml). C (■), AA (150 µM) (○), EPA (150 µM) (▲). n = 6 for C; n = 5 for AA and EPA.

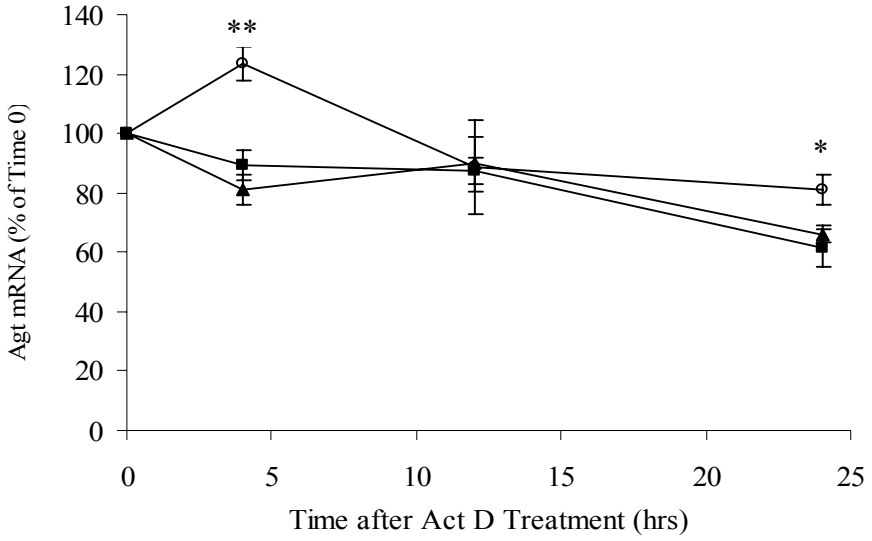


Figure 15: Stability of Agt mRNA in 3T3-L1 Adipocytes Treated with PUFAs

Agt mRNA levels were measured in triplicate in adipocytes treated with PUFAs at 0 hrs, 4 hrs, 12 hrs and 24 hrs following the treatment of actinomycin D (10 μg/ml). The graph represents the average expression values from 2 independent RT-PCR experiments. C (■), AA (○), EPA (▲). n = 7 – 9 for each treatment at each time point. * $P < 0.05$ vs control; * $P < 0.0001$ vs control.

TLR expression and signaling as a potential mechanism of Agt regulation

It is possible that the effects of PUFAs on Agt expression is mediated by TLR signaling. To investigate this possibility, TLR4 protein and mRNA expression were measured. Intracellular protein expression of TLR4 was significantly elevated in adipocytes treated with AA or EPA compared to the control ($P < 0.05$) (**Figure 16**). Conversely, TLR4 mRNA levels were significantly decreased by AA ($P < 0.05$) and EPA ($P < 0.05$) treatments and a trend for decreased TLR4 by combined treatment with AA and EPA ($P < 0.06$) (**Figure 17**).

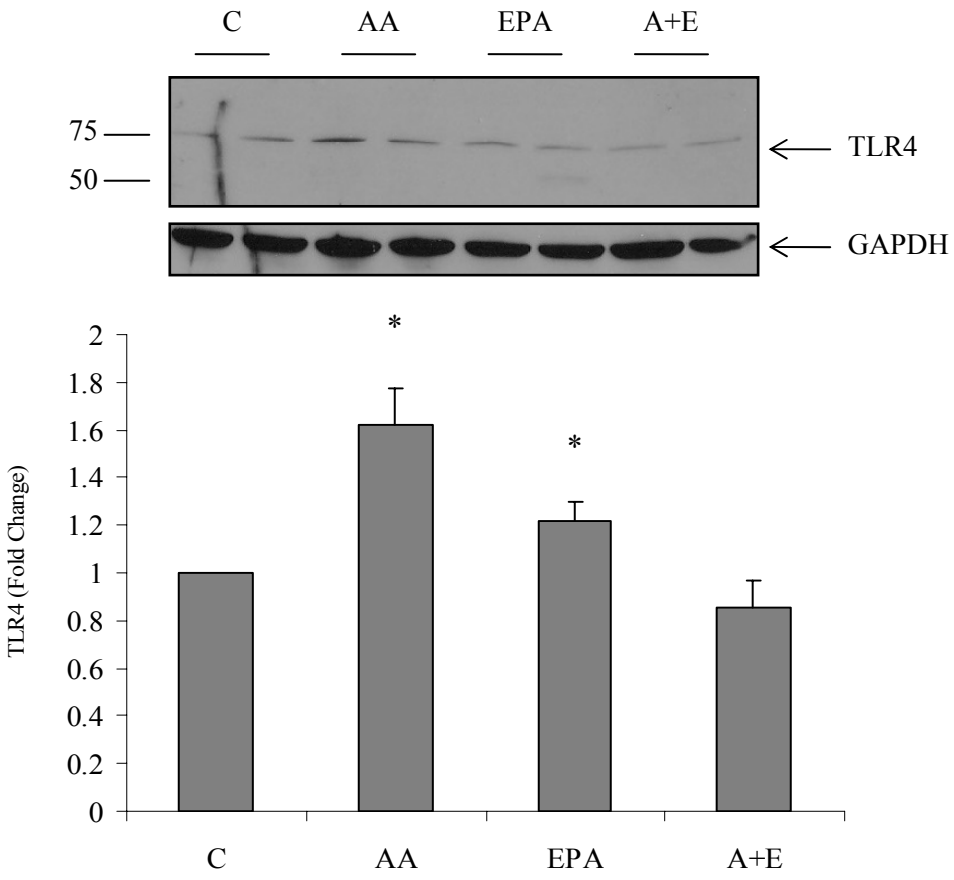


Figure 16: TLR4 Protein Expression in 3T3-L1 Adipocytes Treated with PUFAs

Western blot is representative of 3 different blots. Lanes 1 and 2, Control (C); Lanes 3 and 4, AA (150 μM); Lanes 5 and 6, EPA (150 μM); Lanes 7 and 8, A+E (150 μM AA+EPA). GAPDH was used as a loading control. The approximate molecular weight of TLR4 is 89 kD and GAPDH is 37 kD. The graph shows the relative fold change of TLR4 expression in treated cells vs control. n = 6 for all treatments. * $P < 0.05$ vs control.

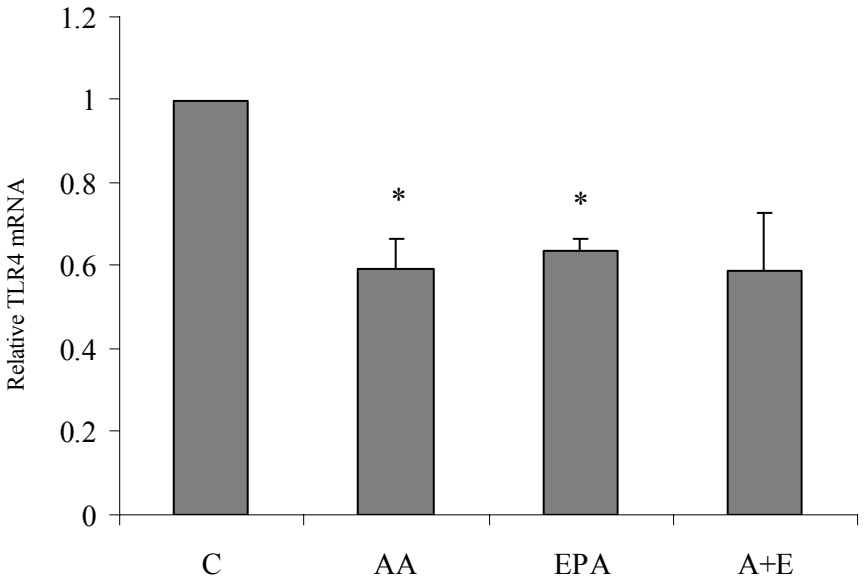


Figure 17: TLR4 mRNA Expression in 3T3-L1 Adipocytes Treated with PUFAs

TLR4 mRNA levels were measured in triplicate in adipocytes treated with 150 μM AA, 150 μM EPA, 150 μM AA+EPA (A+E) or vehicle (C).. The graph represents the average relative expression values from 2 independent RT-PCR experiments. n = 4 for C, AA and A+E; n = 3 for EPA. * $P < 0.05$ vs control.

III. Discussion

Research addressing the dietary regulation of the renin-angiotensin system (RAS) in adipose tissue by polyunsaturated fatty acids (PUFAs) is limited. However, numerous lines of evidence suggest that there is a correlation between adipose RAS, diet, obesity and obesity-related disorders. For example, Agt expression is altered in obese subjects compared to their lean counterparts, with most obese animal models showing higher Agt expression. Agt is also differentially expressed in visceral versus subcutaneous tissue and higher expression levels are present in visceral fat. Further, some PUFAs suppress lipogenic gene expression in adipose tissue and may consequently alter the expression of other adipocyte genes. Studies also indicate that treatment of adipocytes with PUFAs increases Agt expression in a differentiation-dependent

manner. Collectively, these findings suggest that there is a relationship between adipose RAS, obesity and diet.

While previous studies convincingly support a relationship between adipose RAS, obesity and diet, the specific mechanisms of these interactions are largely unknown. To determine if PUFAs differentially regulate Agt expression in adipocytes, protein and mRNA expression levels were measured in 3T3-L1 adipocytes treated with AA, EPA, AA + EPA, or vehicle and their corresponding culture media. Treatment with PUFAs resulted in an increase in intracellular Agt regardless of which fatty acid they were treated with. Further, the n-6 PUFA arachidonic acid (AA) selectively increased Agt secretion into culture media. Interestingly, treatment with increasing doses of EPA appeared to decrease Agt secretion into the media although statistical significance was not reached. This suggests that the beneficial effects of n-3 PUFAs may be dose-dependent. RT-PCR data indicate that AA significantly increases Agt mRNA expression while EPA does not.

Transcription is a major step in the regulation of gene expression. Transcriptional regulation typically involves the interaction of several transcription factors, which bind to target DNA sequences. Agt expression may be regulated by fatty acids at the transcriptional level. Long-chain fatty acids were shown to increase Agt expression in murine preadipocytes by a PPAR-mediated process (110). Adipogenic factors are usually required for terminal differentiation of preadipocytes into adipocytes. However, even in the absence of these factors, fatty acids are still effectively able to activate gene expression, eliminating the possibility that the observed effects of fatty acids are due to differentiation. To determine if PUFAs regulate Agt expression, Agt mRNA levels were measured. Increased Agt mRNA and increased intracellular Agt protein suggests there is an increased production of the RAS precursor which becomes selectively secreted. Expression of the other RAS components, including ACE (**Appendix, Figure A1**), renin (**Appendix, Figure A2**), AT_1R (**Appendix, Figure A3**)and AT_2R (**Appendix, Figure A4**), were also evaluated and results are summarized in the appendix.

To the best of our knowledge, this is the first study showing increased Agt secretion in adipocytes treated with AA. Increased Agt secretion by n-6 PUFAs but not n-3 PUFAs implies that fatty acids differentially regulate adipose RAS. The ability of PUFAs to differentially regulate Agt is significant. Numerous studies have shown that increases in adipose RAS

expression is linked to obesity-related hypertension in humans and in rodents (10, 15, 16, 34, 50, 96, 105, 143). Moreover, it has been suggested that the balance of n-3 to n-6 PUFAs is important for normal physiology and development. Increased intake of n-6 PUFAs coupled with a decrease in n-3 PUFA intake results in the generation of high levels of n-6 derived eicosanoids, including prostaglandins (PGs), thromboxanes, leukotrienes and lipoxins. Large quantities of these eicosanoids induce a prothrombotic state (128). Importantly, secretion mechanisms were not examined to determine if they are differentially modulated by PUFAs. Previous studies have investigated Agt secretion mechanisms in adipocytes (142). However, the effects of PUFAs were not examined. Thus, it is possible that changes in protein synthesis and secretion rate account for the differential effects of PUFAs on Agt in adipocytes.

Preadipocytes are inevitably present even after differentiation, though only in very small amounts. To eliminate the possiblity that the observed results are due to preadipocytes and not to PUFA effects, Agt protein expression was examined in preadipocytes treated with PUFAs. As expected, Agt was detected in mature adipocytes treated with vehicle, which was used as a positive control, but was not detected in preadipocytes. Accordingly, the effects of PUFAs on Agt secretion is not due to preadiocytes.

The cleavage of Agt into Ang I is the rate-limiting step of the RAS pathway. Increased Agt secretion in response to AA could be a secondary result due decreased renin activity, which would result in decreased conversion of Agt into Ang I and, thus, increased secretion of uncleaved Agt. To determine if renin activity is altered by AA, Ang I protein levels were measured in cell lysates and their corresponding culture media following treatment with PUFAs. Renin levels in the media were significantly increased by AA, which may indicate that there is an increase in renin transcription and activity in response to n-6 PUFAs. However, intracellular renin could not be detected likely due to rapid secretion of Ang I and/or rapid intracellular conversion of Ang I into Ang II. Other studies have indicated that although renin expression has been detected in adipose tissue, renin enzymatic levels have not been consistently demonstrated (48, 71, 103).

Increased Agt mRNA levels by AA may be due to increased transcription. However, it may also be due to increased stability of Agt mRNA. Theoretically, the rate of specific transcription, processing, or turnover can modulate mRNA concentration. Gene regulation is

often achieved through the modulation of mRNA stability. Previous studies have shown that mRNA stability can be modulated by PUFAs in adipocytes. SCD1 gene expression is inhibited by PUFAs via decreased mRNA stability (121). Specifically, treatment with AA decreased the half-life of SCD1 mRNA. A similar study in adipocytes showed that GLUT4 mRNA stability is also reduced by treatment with AA (137). To determine if the regulation of adipocyte Agt gene expression by PUFAs is achieved through changes in message stability, total RNA and Agt mRNA levels were assessed over time following the addition of a transcriptional inhibitor, actinomycin D. PUFA-treated adipocytes were subsequently treated with the transcriptional inhibitor, actinomycin D. As expected, total RNA was decreased by both PUFA treatments. Treatment with EPA decreased mRNA stability of Agt while overall, AA increased stability. These findings indicate that n-6 PUFAs differentially stabilize Agt mRNA and provides one possible mechanism by which AA regulates Agt.

It has been demonstrated that TLR4 can regulate energy intake and expenditure as well as mediate cross-talk between the inflammatory and metabolic pathways (60, 73, 135). Indeed, TLR4 expression and activity are higher in the adipocytes and macrophages of high fat diet-induced obese, genetically obese (ob/ob) and diabetic (db/db) mice compared to wild-type littermates (12, 14, 130). TLR4 expression has been shown to localize on the surface of immune cells as well as adipocytes where it binds to bacterial lipopolysaccharide (LPS) and induces nuclear factor-kappa B (NF-κB) translocation into the nucleus resulting in an increase in the transcription of proinflammatory cytokines such as IL-6, TNF-α and COX-2 (37, 89, 102, 136). Recently, it was shown that there is an increase in TLR4 protein expresion following treatment with saturated or monounsaturated fatty acids (117). Significantly, the authors did not measure the effects of PUFAs on TLR4 expression. Our results show for the first time that TLR4 protein expression is significantly increased by both AA and EPA PUFAs. This suggests that fatty acids may increase the production of cytokines via activation of TLRs. Importantly, changes in downstream signaling activity were not measured. It is possible that although expression of TLR4 is increased by PUFAs, signaling may be attenuated.

The increase in TLR4 expression by both n-3 and n-6 PUFAs may be due to nonspecific activation by all fatty acids. Although statistically significant, the changes in TLR4 protein levels were subtle. It is possible that an LPS challenge is necessary to ellicit a greater response in TLR4

expression as it is the ligand activator. Indeed, stimulation with LPS significantly increased TLR2 expression in preadipocytes compared to unchalleged preadipocytes (Claycombe, et al., unpublished data).

Interestingly, we found that TLR4 and TLR2 (**Appendix, Figure A5**) mRNA levels were decreased by PUFAs. Other studies have reported similar results in LPS-treated porcine adipocytes (47). This may represent increased translation of TLR4 into protein although it remains possible that the decrease is a nonspecific effect of fatty acids.

TNF-α can directly modulate lipid metabolism and glucose homeostasis (61, 63, 123). It has been shown to stimulate lipolysis in adipocytes and induce insulin resistance and is directly regulated by NF-κB (64). TNF-α is associated with obesity and insulin resistance and is upregulated in obese individuals. Indeed, obese subjects exhibit extremely high amounts of TNF-α, especially in adipose tissue (62). Significant increases in TNF-α impair insulin sensitivity presumably through the phosphorylation of serine residues and subsequent deactivation of IRS-1.

The alteration of adipokine expression profiles by TLR4 and TLR2 has been implicated as a potential mechanism for fatty acid-induced inflammation and insulin resistance. Indeed, activation of PKC, NF-κB and MAPK signaling pathways lead to increased cytokine production and an inflammatory state in 3T3-L1 adipocytes (20). In mice, palmitate caused greater production of TNF-α and IL-10 in adipocytes compared to OA and DHA (21). To assess the downstream effects of PUFAs on TLR4 signaling, TNF-α and IL-6 intracellular protein and secretion levels were measured. TNF-α levels were undetectable in the media and were not significantly different in the cell compared to the control (**Appendix, Figure A6**). Similarly, IL-6 protein levels in the cell (**Appendix, Figure A7**) and in the media (**Appendix, Figure A8**) were not significantly changed in response to PUFA treatment.

Peroxisome proliferator activated receptors (PPARs) are members of the nuclear receptor superfamily. They are major regulators of adipocyte differentiation. PPARγ is a ligand activated transcription factor. Alternative splicing of PPARγ gives two forms. PPARγ1 is widely expressed by a number of different cell types whereas PPARγ2 is exclusively expressed in adipocytes. PPARγ heterodimerizes with other proteins, including (C/EBP) and 9-cis-retinoic acid receptor (RXR), and this complex binds to response elements within the promoter of target genes to regulate transcriptional activity. Numerous studies have shown that PPAR proteins have

an affinity for PUFAs (9, 79). Indeed, PUFAs are able to bind directly to PPARs and modulate the expression and activity of lipogenic or lypolytic genes such as ADD1/SREBP1c, cAMP response element (CREB) and GATA-binding transcription factors (i.e. GATA2, GATA3) (9).

PPARs were recently shown to have anti-inflammatory effects in adipose tissue. Activation of PPARγ by PUFAs decreases phosphorylation of IκB, which then inhibits NF-κB signaling and the subsequent production of proinflammatory cytokines (87, 100). Thus, the anti-obesity effects of n-3 PUFAs may be due to decreased PPARγ expression (108). However, this remains controversial as other studies have reported induction of PPARγ expression by n-3 PUFAs and stimulation of adipogenesis (17, 79, 92). Our results show that PPARγ protein expression is significantly increased by AA (**Appendix, Figure A9**). Although statistical significance was not reached, treatment with EPA appears to decrease PPARγ protein expression. Thus, PPARγ cannot be eliminated as a potential mediator of PUFA regulation of Agt. Importantly, future studies should take into consideration that PUFAs can bind and activate PPARs, and this is a potentially confounding factor in determining the role of PPARs.

Arachidonic acid is converted into 2-series prostaglandins (PGE_2) and prostacyclins (PGI_2) by the cyclooxygenase complex (COX) and then secreted by adipocytes. Prostoglandins are ligands for the EP receptors present on the cell surface and can act in an autocrine or paracrine manner to increase PGE_2 generation from AA. Adipose tissue secretion of PGE_2 increases FAS enzyme activity and decreases lipolysis in obese individuals by decreasing cAMP levels, and this is mediated through interaction with EP_3 receptors (38). The result is an increase in endogenous fatty acid production. EPA competitively inhibits the generation of PGE_2 from AA and the 3-series prostaglandins (PGE_3) are produced instead. Studies have shown that many of the beneficial effects of PUFAs are mediated by changes in PG levels. Indeed, COX-2 protein expression was inhibited by PUFAs but potentiated by saturated fat via TLR-mediated NF-κB signaling in mouse monocytes (83). Therefore, PUFA regulation of Agt may be mediated by PGs. COX-1 and COX-2 mRNA expression were examined in PUFA-treated adipocytes. COX-1 (**Appendix, Figure A10**) and COX-2 (**Appendix, Figure A11**) mRNA levels were significantly decreased by AA. The significance of this is unclear, however, as COX-1 and COX-2 protein expression were not examined.

To further examine the role of PGs, adipocytes previously treated with the COX-2

inhibitor celecoxib (CEL) were used to assess Agt protein expression in cell lysates (**Appendix, Figure A12**) and corresponding culture media (**Appendix, Figure A13**) following treatement with fatty acids. Consistent with previous results, Agt secretion was significantly increased by AA ($P < 0.001$) and decreased by EPA ($P < 0.0001$) in media samples.

Chapter 4: Conclusions and Future Directions

The purpose of this study was to determine whether fatty acids differentially modulate Agt expression and secretion and to investigate possible mechanism(s) mediating the regulation of adipose RAS in 3T3-L1 adipocytes. We hypothesized that n-3 PUFAs would differentially regulate adipose RAS expression and positively alter adipocyte metabolism, and that mechanisms involved include altered TLRs and/or prostaglandins. Our results are as follows:

1. n-6 but not n-3 PUFAs increase Agt secretion in 3T3-L1 adipocytes.
2. Agt protein levels increase with increasing doses of AA and decrease with increasing doses of EPA.
3. Regulation of Agt secretion is not specific to adipocytes and not due to preadipocytes but may be attributed to changes in renin activity.
4. PUFAs increase mRNA expression of RAS components, especially Agt in AA-treated cells.
5. n-6 PUFAs increase Agt mRNA stability while n-3 PUFAs do not.
6. TLR2 and TLR4 protein expression are both increased by PUFAs.

Significantly, results indicated that treatment with n-3 and n-6 PUFAs increase intracellular Agt while n-6 PUFAs, but not n-3 PUFAs, differentially increase Agt secretion. These effects appear to be dose-dependent as Agt protein levels increased with increasing doses of AA but decreased with increasing doses of EPA. Decreased secretion of Agt by n-3 PUFA further supports the beneficial effects of n-3 fatty acids in metabolic disorders and cardiovascular health. Regulation of Agt by PUFAs was not an effect of preadipocytes but may be due in part to changes in renin activity. Secretion mechanisms were not investigated in this study and should be addressed in future studies. It is important to dissect the mechanisms of secretion to understand why n-6 PUFAs differentially increase secretion while n-3 PUFAs do not.

Results also showed that PUFAs increase mRNA expression of RAS components, including Agt, ACE and renin, with AA having the greatest effect on Agt expression. In addition to regulating transcription, AA also increases Agt mRNA stability while EPA does not. Thus, it

appears that mechanisms of regulation of Agt by PUFAs are complex. Future studies are needed to clarify the mechanisms mediating PUFA effects on Agt in adipocytes and to identify putative transcription factors involved in transcriptional regulation.

TLR4 protein expression was increased by PUFAs suggesting that it may be involved in the regulation of adipocytes. Importantly, we did not stimulate cells with LPS, which may account for the low TLR protein expression levels that were observed. Thus, stimulation of PUFA-treated adipocytes with LPS is necessary for examinations of TLR expression in adipocytes as a possible mediator of Agt regulation. In order to better understand whether TLR signaling is involved in PUFA regulation, downstream signaling molecules should be investigated, including PI3K and NF-κB. Though we examined IL-6 and TNFα cytokines in this study, it may be useful to perform multiplex cytokine analysis to better understand how the inflammatory response is altered by PUFAs in adipocytes. Numerous lines of evidence support a role for innate immunity in the development of obesity-related inflammation and insulin resistance and this remains an interesting and novel area of research, particularly in adipocytes.

Preliminary studies were conducted to address other potential mechanisms that may mediate PUFA regulation of Agt in adipocytes such as PPARs or prostaglandins. We were unable to obtain consistent results for PPARs using existing samples. It seems likely that this was due to sample quality and studies should be repeated. PPAR agonists and antagonists can be used to facilitate investigations of the role of PPARs in Agt regulation. PPARs are able to bind to a variety of substrates making it difficult to draw conclusions. Consequently, experiments should be carefully designed with this in mind. Treatment studies with the COX-2 inhibitor celecoxib and PUFAs and the subsequent examination of Agt will facilitate further investigation of the role of prostaglandins in mediating Agt regulation by PUFAs.

While additional experiments are clearly needed to further dissect the mechanisms mediating regulation of Agt and other RAS genes by PUFAs, our studies demonstrate for the first time that n-6 PUFAs specifically increase Agt secretion while n-3 PUFAs decrease secretion. These findings support the beneficial effects of n-3 fatty acids in metabolic disorders and cardiovascular health.

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List of References

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Appendix

Introduction

Several mechanisms may mediate the effects of PUFAs on Agt in adipocytes. One possibility is that prostaglandins mediate this regulation. Studies have shown that many of the beneficial effects of PUFAs are mediated by changes in PG levels. PPARγ plays a central role in regulating adipogenesis and may also be involved in PUFA regulation of Agt. Further, increasing evidence supports a potentially significant role of the innate immune system in the etiology of obesity-related metabolic diseases via inflammatory signaling pathways, specifically the toll-like receptor 4 (TLR4) signaling pathways. Indeed, Ang II activates many of the same pathways as TLR4. However, it is not know whether TLRs can mediate the effects of PUFAs on Agt expression in adipocytes. Thus, additional experiments were performed to further investigate potential mechanisms underlying the regulation of Agt by PUFAs. Related findings have also been referenced in the discussion section of this thesis.

Materials and Methods

Samples were obtained from treatment studies previously performed by our lab using the COX-2 inhibitor celecoxib (CEL) or PUFAs (148). Briefly, 3T3-L1 preadipocytes were cultured and differentiated as described above. 24 hours prior to treatment, starvation media was added. Fatty acids were solubilized in dimethyl sulfoxide (DMSO) and then conjugated to fatty acid-free BSA by agitation in a 37°C water bath for 2 hours prior to treatment. Cells were treated with 5 μM Celebrex (CEL) (Pharmacia, St. Louis, MO), 150 μM OA, 150 μM AA, 150 μM EPA, 150 μM AA+EPA (75 μM of each fatty acid), or vehicle (DMSO) for 48 hours.

Results

The effects of PUFAs on Agt (thesis main results) as well as other RAS components were assessed in adipocytes. There were increased ACE mRNA levels in EPA-treated cells ($P < 0.05$) (**Figure A1**). Renin mRNA expression was not significantly different in treated versus control cells (**Figure A2**). AT_1R mRNA levels were significantly decreased in cells treated with EPA compared to the control ($P < 0.05$) (**Figure A3**). AT_2R mRNA levels were decreased by all PUFA treatments ($P < 0.05$) (**Figure A4**).

TLR2 can also bind fatty acids and activate the same pathways as TLR4. Thus, TLR2

was assessed in PUFA-treated adipocytes. The effects of PUFAs on TLR2 mRNA expression appears to be differential and AA decreases TLR2 ($P < 0.01$) while EPA and AA+EPA do not significantly alter levels (**Figure A5**). To assess whether the increase in TLR4 protein expression has an effect on downstream events, intracellular and secreted TNF-α and IL-6 were measured by ELISA. Intracellular TNF-α levels were not significantly different from control treated samples (**Figure A6**) and were undetectable in the media. Similarly, both intracellular IL-6 (**Figure A7**) and IL-6 secretion (**Figure A8**) in PUFA-treated samples were similar to the control.

PUFAs can bind directly to PPARγ and alter lipogenic or lipolytic gene expression resulting in decreased inflammation. To determine if the beneficial effects of n-3 PUFAs on Agt are mediated by PPARγ, protein and mRNA levels were measured. Unexpectedly, PPARγ protein expression was significantly increased by AA ($P < 0.05$) but unchanged by EPA or combined treatment with AA + EPA (**Figure A9**).

The COX enzyme complex catalyzes the first step in the generation of prostaglandins, prostacyclins and thromboxanes. PUFA regulation of Agt in adipocytes may be mediated by prostaglandin levels. Thus, COX-1 and COX-2 expression and activity were examined in adipocytes treated with PUFAs. COX-1 mRNA ($P < 0.05$) (**Figure A10**) and COX-2 mRNA ($P < 0.01$) (**Figure A11**) levels were both decreased by treatment with AA.

To investigate whether prostaglandins mediate the effects of PUFAs on Agt, adipocytes were treated with CEL, OA, AA, EPA, or vehicle. Agt protein expression in cell lysates (**Figure A12**) and media samples (**Figure A13**) following treatment was examined. Consistent with previous results, Agt secretion was significantly decreased in media samples from cells treated with EPA and significantly increased by treatment with AA. Effects on intracellular Agt were inconclusive, likely due to sample degradation. The effects of the COX-2 inhibitor celecoxib (CEL) on Agt expression were also inconsistent and further studies are needed.

Adiponectin is an important marker of insulin sensitivity and in general adiponectin levels are inversely related to adiposity. The effects of PUFAs on insulin sensitivity were assessed by measuring adiponectin secretion. Treatment with CEL significantly increased adiponectin secretion ($P < 0.05$) (**Figure A14**). In contrast, adiponectin secretion was significantly decreased by treatment with AA ($P < 0.001$). Decreased adiponectin by AA suggests an increased risk for obesity and insulin resistance by n-6 PUFAs but not n-3 PUFAs.

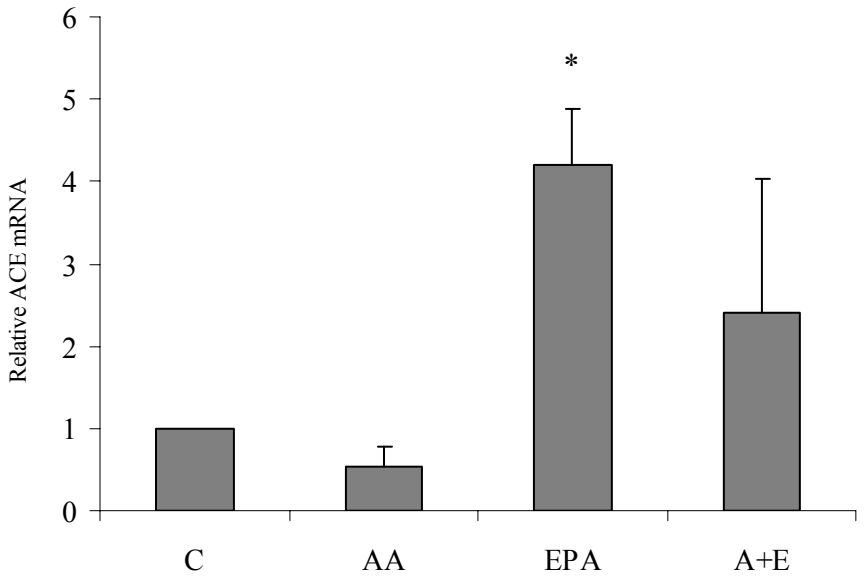

Figure A1. ACE mRNA Expression in 3T3-L1 Adipocytes Treated with PUFAs

ACE mRNA levels were measured in triplicate in adipocytes treated with 150 μM AA, 150 μM EPA, 150 μM AA+EPA (A+E) or vehicle (C). The graph represents the average expression values from 3 independent RT-PCR experiments. n = 4 for C; n = 3 for AA, EPA and A+E. * $P < 0.05$ vs control.

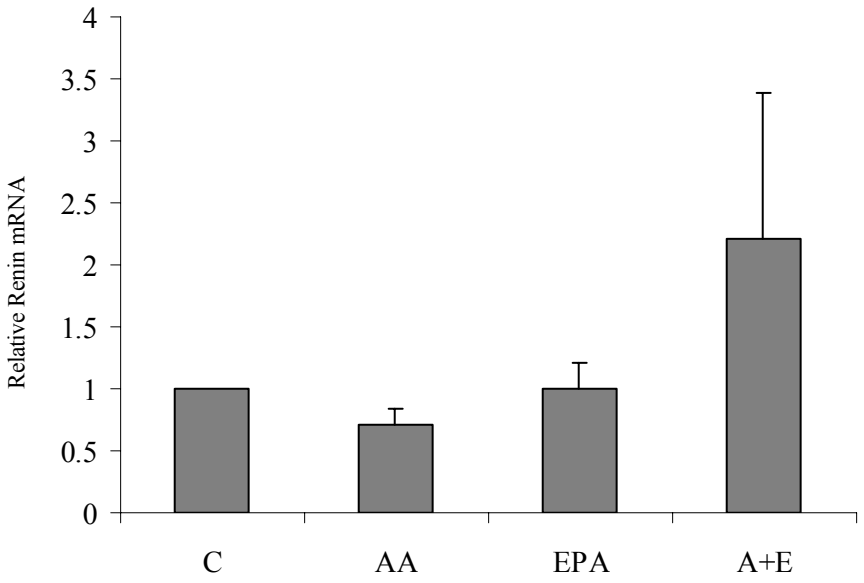

Figure A2. Renin mRNA Expression in 3T3-L1 Adipocytes Treated with PUFAs

Renin mRNA levels were measured in triplicate in adipocytes treated with 150 μM AA, 150 μM EPA, 150 μM AA+EPA (A+E) or vehicle (C). The graph represents the average expression values from 3 independent RT-PCR experiments. n = 4 for C, AA and A+E; n = 3 for EPA.

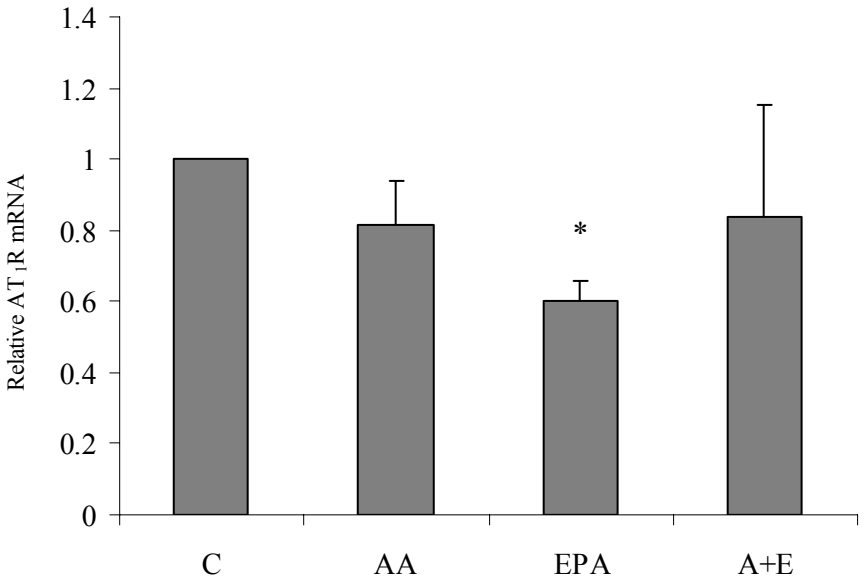

Figure A3. AT_1R mRNA Expression in 3T3-L1 Adipocytes Treated with PUFAs

AT_1R mRNA levels were measured in triplicate in adipocytes treated with 150 μM AA, 150 μM EPA, 150 μM AA+EPA (A+E) or vehicle (C). The graph represents the average expression values from 2 independent RT-PCR experiments. n = 4 for C, AA and A+E; n = 3 for EPA. * $P < 0.05$ vs control.

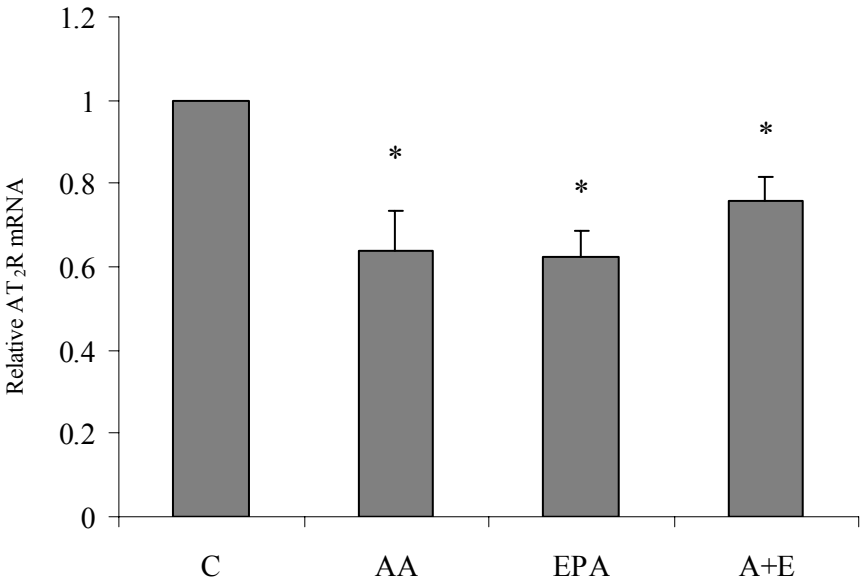

Figure A4. AT_2R mRNA Expression in 3T3-L1 Adipocytes Treated with PUFAs

AT_2R mRNA levels were measured in triplicate in adipocytes treated with 150 μM AA, 150 μM EPA, 150 μM AA+EPA (A+E) or vehicle (C). The graph represents the average expression values from 3 independent RT-PCR experiments. n = 4 for C, AA and A+E; n = 3 for EPA. * $P < 0.05$ vs control.

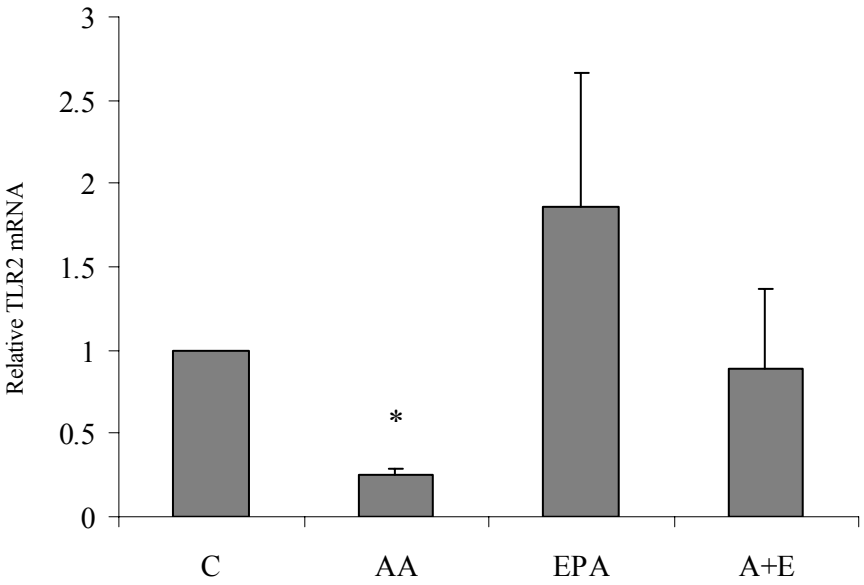

Figure A5. TLR2 mRNA Expression in 3T3-L1 Adipocytes Treated with PUFAs

TLR2 mRNA levels were measured in triplicate in adipocytes treated with 150 μM AA, 150 μM EPA, 150 μM AA+EPA (A+E) or vehicle (C). The graph represents the average expression values from 2 independent RT-PCR experiments. n = 4 for C; n = 3 for AA, EPA and A+E. * $P < 0.01$ vs control.

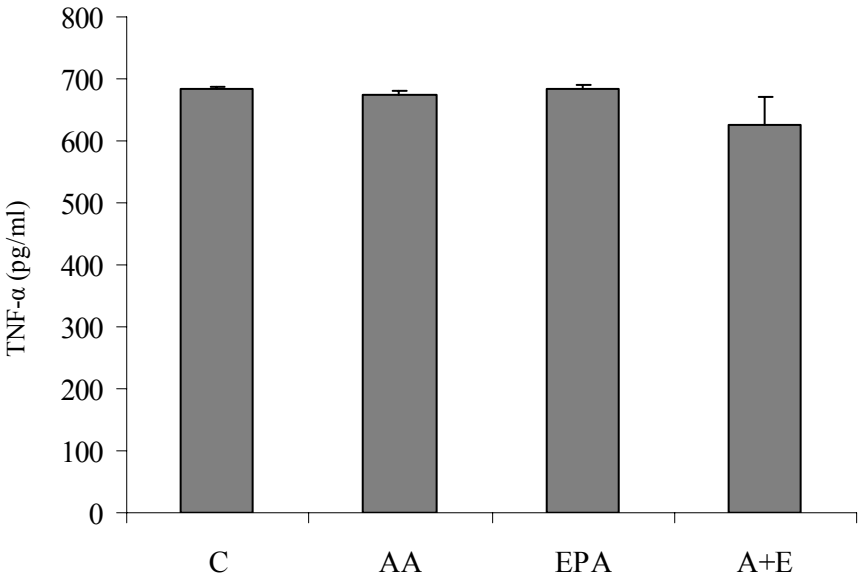

Figure A6. TNF-α Protein Levels in Culture Media of 3T3-L1 Adipocytes Treated with PUFAs

TNF-α protein levels were measured in the culture media of adipocytes treated with 150 μM AA, 150 μM EPA, 150 μM AA+EPA (A+E) or vehicle (C). n = 6 for all treatments.

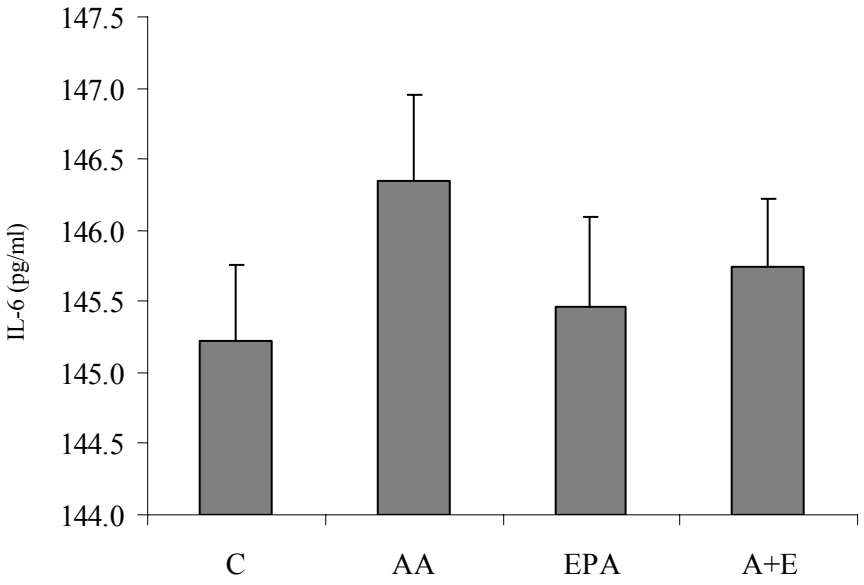

Figure A7. IL-6 Protein Levels in 3T3-L1 Adipocytes Treated with PUFAs

IL-6 protein levels were measured in adipocytes treated with 150 μM AA, 150 μM EPA, 150 μM AA+EPA (A+E) or vehicle (C). n = 6 for all treatments.

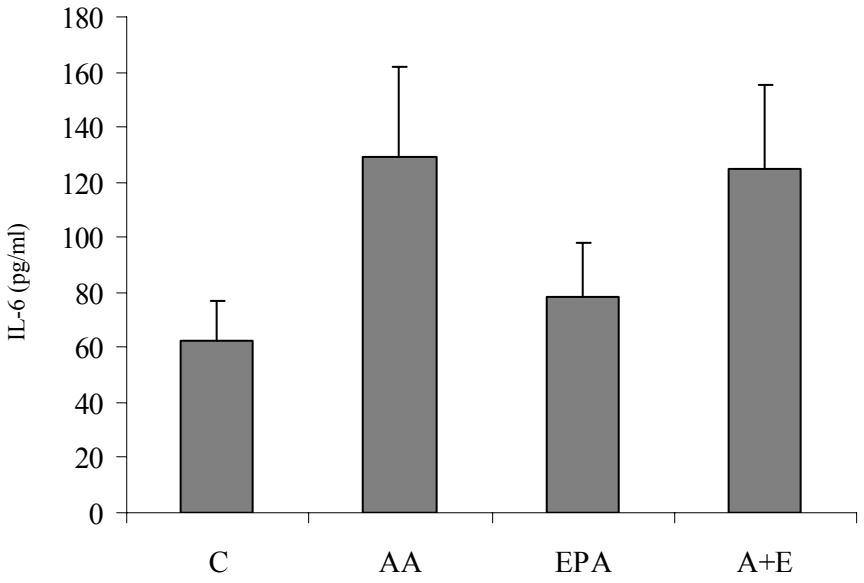

Figure A8. IL-6 Protein Levels in Culture Media of 3T3-L1 Adipocytes Treated with PUFAs

IL-6 protein levels were measured in the culture media of adipocytes treated with 150 μM AA, 150 μM EPA, 150 μM AA+EPA (A+E) or vehicle (C). n = 8 for all treatments.

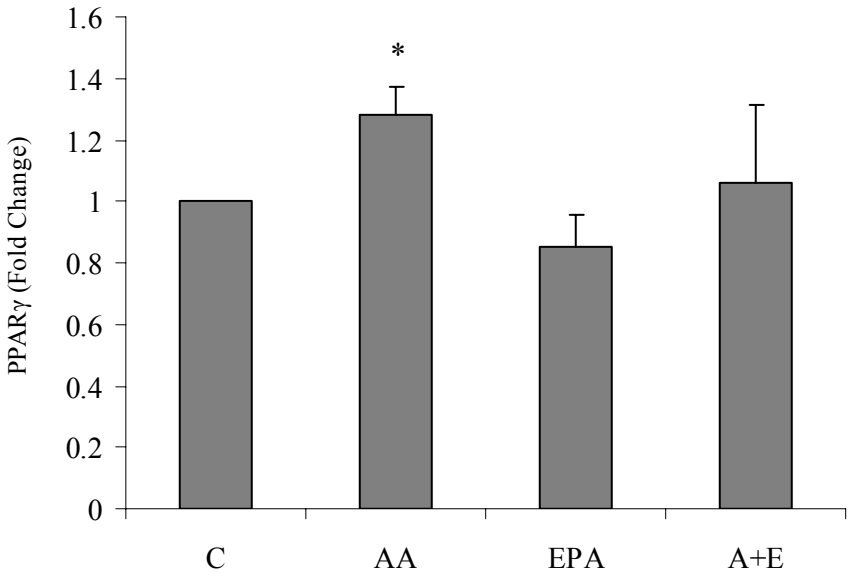

Figure A9. PPARγ Protein Expression in 3T3-L1 Adipocytes Treated with PUFAs

PPARγ protein expression was assessed in cell lysates in response to treatment with 150 μM AA, 150 μM EPA, 150 μM AA+EPA (A+E) or vehicle (C). The graph shows relative fold change of PPARγ expression in treated cells vs control. n = 4 for all treatments. * $P < 0.05$ vs control.

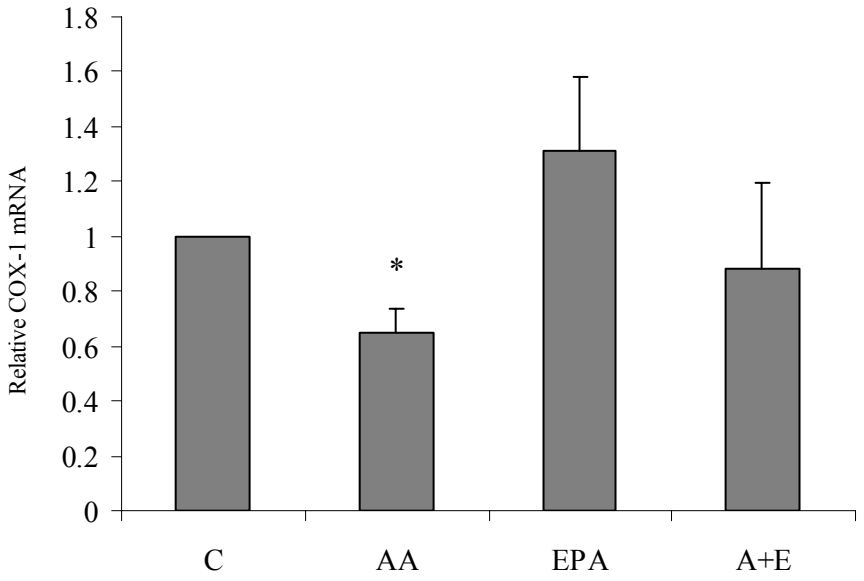

Figure A10. COX-1 mRNA Expression Levels in 3T3-L1 Adipocytes Treated with PUFAs

COX-1 mRNA levels were measured in triplicate in adipocytes treated with 150 μM AA, 150 μM EPA, 150 μM AA+EPA (A+E) or vehicle (C). The graph represents the average expression values from 3 independent RT-PCR experiments. n = 4 for C; n = 3 AA, EPA and A+E. * $P <$ 0.05 vs control.

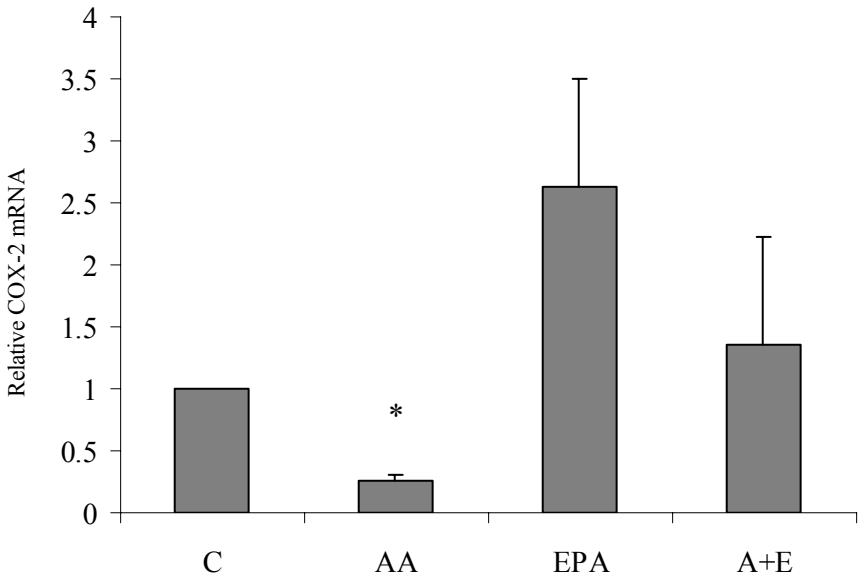

Figure A11. COX-2 mRNA Expression Levels in 3T3-L1 Adipocytes Treated with PUFAs

COX-2 mRNA levels were measured in triplicate in adipocytes treated with 150 μM AA, 150 μM EPA, 150 μM AA+EPA (A+E) or vehicle (C). The graph represents the average expression values from 3 independent RT-PCR experiments. n = 4 for C; n = 3 AA, EPA and A+E. * $P <$ 0.01 vs control.

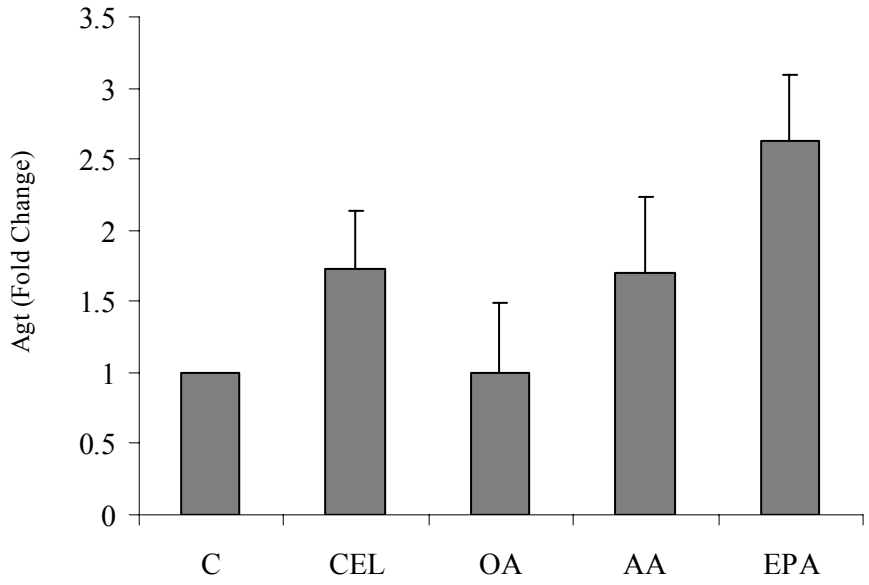

Figure A12. Agt Protein Expression in 3T3-L1 Adipocytes Treated with PUFAs

Agt protein expression was assessed in adipocytes treated with 5 μM Celecoxib (CEL), 150 μM OA, 150 μM AA,150 μM EPA or vehicle (C). The graph shows relative fold change of Agt expression in treated cells vs control. n = 6 for all treatments. * $P < 0.01$ vs control.

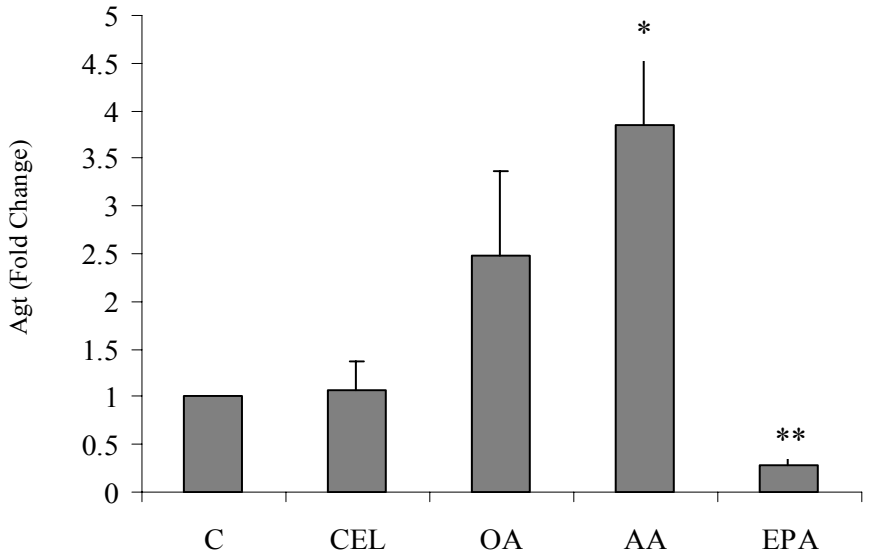

Figure A13. Agt protein expression in Culture Media of 3T3-L1 Adipocytes Treated with PUFAs

Agt protein expression was assessed in the culture media of adipocytes treated with 5 μM Celecoxib (CEL), 150 μM OA, 150 μM AA,150 μM EPA or vehicle (C). The graph shows relative fold change of Agt expression in treated cells vs control. n = 10 for C; n = 4 for CEL and OA; n = 14 for AA; n = 9 for EPA. * $P < 0.001$ vs control; ** $P < 0.0001$ vs control.

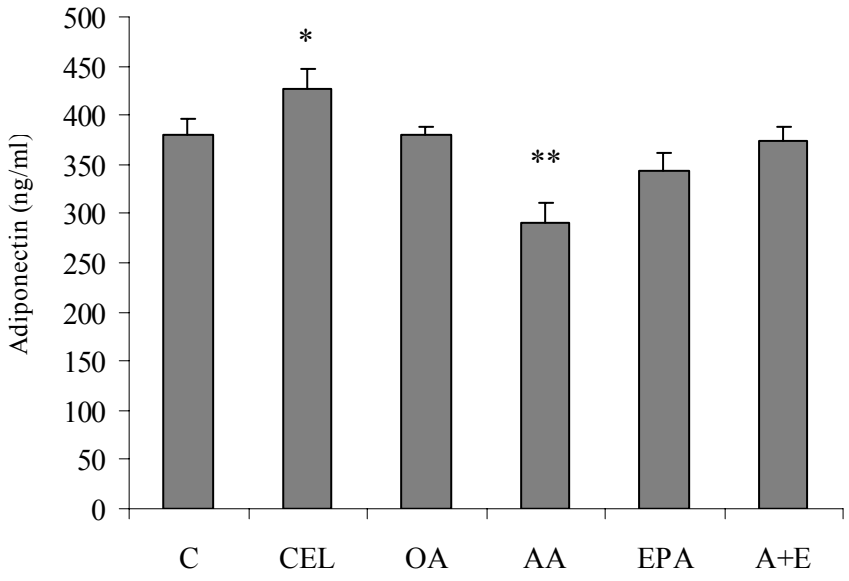

Figure A14. Adiponectin Protein Levels in Culture Media of 3T3-L1 Adipocytes Treated with PUFAs

Adiponectin protein levels were measured in the culture media of adipocytes treated with 5 μM Celecoxib (CEL), 150 μM OA, 150 μM AA,150 μM EPA, A+E (150 μM AA+EPA) or vehicle (C). n = 10 for all treatments. * $P < 0.05$ vs control; ** $P < 0.001$ vs control.

Summary

Potential mechanisms mediating the regulation of Agt by PUFAs were examined, including TLRs, PPARγ, or prostaglandins. Other RAS components were also assayed to provide a comprehensive evaluation of adipose RAS expression and activity in response to treatment with PUFAs. While preliminary data was obtained from these experiments, additional studies are needed to clarify the role of these mechanisms in mediating PUFA regulation of Agt in adipocytes.

Vita

Sarah Jean Fletcher was born in Hampton, VA on April 4, 1982. She grew up in a military household and spent the majority of her childhood overseas. She attended elementary school in Pusan, South Korea, Taegu, South Korea, Chesterfield, Virginia, Rotterdam, The Netherlands, and Cleveland, Tennessee. She attended Cleveland Middle School and Cleveland High School where she graduated with honors. Upon graduation, she enrolled in the University of Tennessee where she completed a bachelors of science degree in biochemistry and cellular and molecular biology in 2005 and a masters of science degree in genome science and technology with a minor in statistics in 2009.